

CLINICAL AND METABOLIC STUDIES OF
HUMAN β -HEXOSAMINIDASE

BY

ANDERS ISAKSSON

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Abstract The present study focused on the effects of several well-known diseases, and pregnancy on the activities of the lysosomal enzymes. Particular attention was paid to the serum levels and the metabolism of β-hexosaminidase. The increases of β-hexosaminidase activity in liver diseases (liver cirrhosis, hepatitis and cholestasis) and chronic alcoholism are attributed to liver dysfunction. The increases of enzyme activity during pregnancy and thyrotoxicosis are suggested to be due to hormonal effects. Possible reasons for the slight increases observed in diabetes mellitus and inflammatory bowel diseases are given. β-Hexosaminidase displays enzyme multiplicity. The isoenzymes are denominated as A, B, I₁, I₂, P and S. The isoenzyme pattern was similar in pregnancy, liver diseases and chronic alcoholism, with a preferential increase of the P form. This suggested that a common mechanism was responsible for the changes observed. Although related, the tissue and serum forms of β-hexosaminidase differ in physicochemical properties. Experimental evidence shows that the tissue forms are cleared more rapidly from circulation than the serum forms after intravenous infusion into rats. The clearance of the serum forms is enhanced after desialylation. Desialylated serum forms (A and B, but not P) and tissue forms are taken up by non-parenchymal liver cells (Kupffer and endothelial cells). The tissue forms were not taken up by peritoneal macrophages suggesting that the receptor functions of peritoneal and liver macrophages (Kupffer cells) differ. The clinical usefulness of serum β-hexosaminidase to monitor liver damage and endotoxemia are discussed. Key words β-Hexosaminidase, lysosomal hydrolases, isoenzymes, liver disease, liver cirrhosis, alcohol, chronic alcoholism, pregnancy, clearance, uptake, non-parenchymal liver cells, macrophages, hepatocytes.			
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Distribution by (name and address) Anders Isaksson, Inst.f.klinisk kemi
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CLINICAL AND METABOLIC STUDIES OF
HUMAN β -HEXOSAMINIDASE

AKADEMISK AVHANDLING

av

ANDERS ISAKSSON
Leg läkare, Ld

Offentlig disputation för medicine doktorsgraden
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ERRATA

Paper I, page 320, Table III.

ASAT/Bilirubin: $r=0.22$ should read $r=0.25$.
 $t=1.6$ should read $t=1.8$.

ALAT/Bilirubin: $r=0.22$ should read $r=0.26$.
 $t=1.6$ should read $t=1.8$.

Paper VII, page 232, left column, 8:th line from below.

40 mol/l should read 40 mmol/l.

Paper VII, page 237.

Reference 1 should read: Achord, D. I.; Brot, F. E.; Sly, W. S.: Inhibition of the rat clearance system for agalacto- α -D-glucosaminidase by yeast mannans and by mannose. Biochem. Biophys. Res. Commun. 77:409-415 (1977).

Paper VIII, page 5, 12:th line from below.

-hexosaminidase should read β -hexosaminidase.

Paper VIII, page 10, 8:th line.

-hexosaminidase should read β -hexosaminidase.

Paper VIII, page 14.

Reference 5 should read: Stahl, P. D., Six, H., Rodman, J. S., Schlesinger, P., Iuliani, D. R. P., Touster, O. (1976) Proc. Natl. Acad. Sci. USA 73, 4045-4049



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HUMAN β -HEXOSAMINIDASE

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ANDERS ISAKSSON

Department of Clinical Chemistry
University Hospital
S-221 85 Lund, Sweden

LUND 1985

To
Marléne, Johannes and Dag

This thesis is a summary of the following papers, which will be referred to by Roman figures.

- I Hultberg, B., Isaksson, A. and Tiderström, G. (1980) β -Hexosaminidase, leucine aminopeptidase, cystidyl aminopeptidase, hepatic enzymes and bilirubin in serum of chronic alcoholics with acute ethanol intoxication. Clin. Chim. Acta 105, 317 - 323.
- II Hultberg, B., Isaksson, A. and Jansson, L. (1981) β -Hexosaminidase in serum from patients with cirrhosis and cholestasis. Enzyme 26, 296 - 300.
- III Hultberg, B., Braconier, J. H., Isaksson, A. and Jansson, L. (1981) β -Hexosaminidase level in serum from patients with viral hepatitis as a measure of reticuloendothelial function. Scand. J. Infect. Dis. 13, 241 - 245.
- IV Isaksson, A., Blanche, C., Hultberg, B. and Joelsson, B. (1985) Influence of ethanol on the human serum level of beta-hexosaminidase. Enzyme 33, 162 - 166.
- V Hultberg, B. and Isaksson, A. (1983) Isoenzyme pattern of serum β -hexosaminidase in liver disease, alcohol intoxication and pregnancy. Enzyme 30, 166 - 171.
- VI Isaksson, A., Gustavii, B., Hultberg, B. and Masson, P. (1984) Activity of lysosomal hydrolases in plasma at term and post partum. Enzyme 31, 229 - 233.
- VII Isaksson, A., Hultberg, B., Sundler, R. and Åkesson, B. (1983) Uptake of β -hexosaminidase by nonparenchymal liver cells and peritoneal macrophages. Enzyme 30, 230 - 238.
- VIII Isaksson, A., Bergenfeldt, M., Hultberg, B. and Joelsson, B. Metabolism of the human serum β -hexosaminidase isoenzymes in the rat. An in vivo and in vitro study. Submitted.

ABBREVIATIONS

ASAT	Aspartate aminotransferase (L-Aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1)
ALAT	Alanine aminotransferase (L-Alanine:2-oxo-glutarate aminotransferase, EC 2.6.1.2)
ALP	Alkaline phosphatase (Orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1)
GT	γ -Glutamyltransferase (Glutamine:D-glutamyl-peptide glutamyltransferase, EC 2.3.2.1)
Hex	β -Hexosaminidase, β -N-Acetylglucosaminidase (2-Acetamido-2-deoxy- β -D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30)
α -Fucosidase	α -L-Fucosidase (α -L-Fucoside fucohydrolase, EC 3.2.1.51)
β -Glucuronidase	β -D-Glucuronidase (β -D-Glucuronide glucuronohydrolase, EC 3.2.1.31)
α -Hexosaminidase	α -N-Acetylglucosaminidase (2-Acetamido-2-deoxy- α -D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.50)
α -Mannosidase	α -D-Mannosidase (α -D-Mannoside mannohydrolase, EC 3.2.1.24)

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BACKGROUND

The lysosomal concept

Lysosomes were discovered about thirty years ago (1). Essentially, the lysosomal concept rests on two biochemical criteria:

- 1 The association of a variety of acid hydrolases with a specialized group of cytoplasmic organelles characterized by their centrifugal properties.
- 2 The structure linked latency of the acid hydrolases.

Even in morphological work, lysosomes are identified on the basis of these criteria. The number of lysosomal enzymes currently known is fifty (2), and lysosomes, in one form or another, are now considered a constant feature of all eukaryotic cells in both the animal and plant kingdom.

Biosynthesis and transport of lysosomal enzymes

Our present knowledge refers almost exclusively to soluble enzymes and is limited to connective tissue type cells (3) and macrophages (4). Briefly, a lysosomal enzyme is synthesized as a precursor with a large molecular mass. Like secretory and membrane glycoproteins, the synthesis of the lysosomal enzyme occurs at the rough endoplasmic reticulum.

The biosynthesis of the oligosaccharide moiety, involving a lipid-linked intermediate, has been the subject of recent reviews (5, 6). Initially, the oligosaccharide chains are mannose-rich when transferred from the lipid-linked intermediates to the nascent glycoproteins entering the cisternal space of the endoplasmic reticulum. In the case of secretory glycoproteins, these chains might undergo a series of processing reactions; first they are trimmed down to smaller core oligosaccharides and then rebuilt into more complex chains by the action of several "terminal" glycosyltransferases. A prerequisite for the further processing of the lysosomal enzyme is the phosphorylation of mannose residues in the mannose-rich oligosaccharide to mannose-6-phos-

phate (Man-6-P). This phosphorylation is achieved by a transfer reaction involving N-acetyl-glucosamine-1-phosphate (6, 7, 8).

A specific intracellular membrane receptor binds the precursors containing Man-6-P and collects them into specialized vesicles in the Golgi complex or GERL (9). These bud off as primary lysosomes. An acidic pH in the lysosomes favours the dissociation of the receptor from the enzyme precursors. The receptor is recycled to the Golgi complex for reutilization and the enzyme precursor delivered to secondary lysosomes, where the maturation process involving dephosphorylation and probably proteolysis is completed (3). The half-lives of these mature enzymes vary from several days to several weeks (10, 11).

Although most of the work with this receptor has been performed with fibroblasts, the system appears to be present in all mammalian cells (12). In cell cultures, a small fraction of the lysosomal enzyme precursors may escape segregation into lysosomes and are secreted into the extracellular medium (3, 11). These precursors gain entry into the secondary lysosomes by a second pathway involving pinocytosis mediated by cell surface receptors for Man-6-P. This pathway explains the uptake of corrective factors by enzyme deficient fibroblasts (9). Quantitatively, this pathway is less important than the intracellular one.

Phosphorylation of the mannose residues on the lysosomal enzyme precursor therefore plays a vital role in governing the destiny of the enzyme. In its presence, the precursor is directed to the secondary lysosome and attains maturation. In the absence of phosphorylation, the precursor escapes internalization and is secreted in a form where the oligosaccharide side chain has been modified as in the case of secretory glycoproteins (9).

Endocytosis

Endocytosis is achieved either by phagocytosis or by pinocytosis (13). Phagocytosis involves the internalization of a large particle or molecular aggregate in a correspondingly large vacuole. The process is initiated when a substrate is attached to the plasma membrane.

Pinocytosis is either non-adsorptive or adsorptive. In the case of non-adsorptive pinocytosis, the substrates enter the cell in fluid phase. Adsorptive pinocytosis is related to non-specific adsorption of the substrate to the cell membrane due to hydrophobic or cationic properties or to specific adsorption. In the latter case, pinocytosis occurs only in response to a ligand attaching to a receptor on the cell surface. These receptors are mainly glycoproteins and they cluster in areas that invaginate into pits when combined with specific ligands, eventually detaching as discrete vesicles. Kinetically, non-adsorptive pinocytosis displays a linear relationship for substrate uptake, whilst receptor-mediated pinocytosis follows typical Michaelis-Menten kinetics.

Materials ingested by endocytosis are temporarily stored in digestively inactive vacuoles. These vacuoles (renamed receptosomes or endosomes) are in reality important sorting centers from which materials may selectively be routed to one of three directions: the lysosomes, the cytosol or the extra-cellular space (2).

Under normal circumstances, an acidic pH around 4.5 is maintained in the lysosomal compartment by the action of an energy-dependent proton pump. This pump couples the hydrolysis of ATP to the translocation of protons into the lysosomes (14). The initial effect of lysosomotropic agents such as ammonium chloride and chloroquine that are taken up by lysosomes, is to raise the endo-lysosomal pH to around 6.5. This process is completed in a matter of minutes and is accompanied by a rapid accumulation of the substrate. After pH has stabilized, substrate accumulation continues but at a slower rate. The increase of endo-lysosomal substrate concentration initiates osmosis, causing a progressive expansion of this compartment. This leads to the extensive vacuolation that is seen an hour or more after the onset of endocytosis.

Secretion of lysosomal enzymes

Usually, less than 20 % of the lysosomal enzymes synthesized de novo in cell cultures are secreted to the extracellular medium (3). A minor proportion of these enzymes are precursors with phosphorylated mannose residues. The major fraction consists of precursors either with non-phosphorylated mannose-rich oligosaccharide chains or enzymes with complex oligosaccharide chains terminating in sialic acid residues (15). Mature forms of lysosomal enzymes do not seem to be secreted by fibroblasts, smooth muscle cells, endothelial cells, lymphocytes and hepatocytes (4, 11, 16). However, macrophages are known to release mature lysosomal enzymes upon stimulation (4).

Ammonium-chloride and chloroquine have proven to be invaluable tools in the study of cytophysiological mechanisms of secretion. These compounds alter the intra-lysosomal pH in primary lysosomes to a more basic pH, and prevent the dissociation of Man-6-P containing enzyme precursor from its receptor. This affects the recycling and reutilization of the receptor and causes secretion of newly synthesized enzyme precursors. Hence, dysfunctions in the Man-6-P receptors, Man-6-P ligands, recycling and inhibition of glycosylation all favour secretion of precursors (12, 17).

In contrast to fibroblasts, the macrophages play a major role in secretion, particularly at sites of inflammation. This secretion process is not fully understood, despite the fact that the secretion rate of newly synthesized enzymes is very high in activated macrophages (11, 18). It is known that more compounds induce secretion in macrophages than in fibroblasts. These include complement components, immune complexes, particulate agents such as zymosan and asbestos, and pharmacological compounds such as cytochalasin B and ammonium chloride (19). When exposed to opsonized zymosan, the macrophages release a large portion of the cellular content quite rapidly (within one to four hours). It is possible that the enzymes released are preformed enzymes from existing lysosomes (20). If the macrophages are exposed to zymosan for 1 - 2 days, they continue to secrete up to three times the quantity of enzymes compared to non-stimulated cells, suggesting that newly synthesized enzymes are secreted directly (20, 21).

Since the Man-6-P dependent sorting system exists even in macrophages, the effects of weak bases such as ammonium chloride are similar to those outlined earlier (22). In addition, macrophages have a mannosyl/N-acetylglucosamine glycoprotein receptor which can mediate the clearance of mannose containing glycoproteins. It is possible that this system may even function intracellularly in retention and transport of the lysosomal enzymes (17). Dysfunction in this system may favour secretion.

Clearance of lysosomal enzymes from circulation

The mannosyl/N-Acetylglucosamine glycoprotein receptor present on the cell surface of mononuclear phagocytes (Kupffer cells of the liver, alveolar macrophages and other fixed tissue macrophages such as those of spleen or bone marrow) mediates the uptake of many purified lysosomal enzymes (tissue forms) in vitro. It is likely that the same receptor is responsible for the clearance of the enzymes from plasma following intravenous infusion (23). Present evidence supports the concept that cell surface receptors bearing ligands are rapidly internalized and that receptors are recycled. The liver is primarily responsible for this clearance but spleen and bone marrow also participate to a limited extent (24). Cell fractionation, autoradiographic and histochemical studies with liver have indicated that non-parenchymal cells (i.e. Kupffer cells and endothelial cells) are the principal cell types engaged in the recognition of mannosyl/N-acetylglucosamine terminated glycoproteins (25, 26, 27).

The second receptor that could participate in the clearance is the galactosyl glycoprotein receptor on hepatocytes (28). It has been claimed that this receptor mediates clearance of glycoproteins with complex-type oligosaccharide chains (29).

AIMS OF THE PRESENT INVESTIGATION

Lysosomal hydrolases are most frequently studied in connection with inborn errors of metabolism, where enzyme defect or deficiency results in accumulation of substrate within the lysosomes. The present study was, however, concentrated on the effects of several well-known diseases and pregnancy on the activities of lysosomal enzymes. Particular attention was paid to the enzyme β -hexosaminidase (2-Acetamido-2-deoxy- β -D-glucoside acetamido-deoxyglucohydrolase, EC 3.2.1.30, abbreviated Hex). This enzyme is involved in the degradation of glycoproteins, glycosaminoglycans and sphingolipids and displays enzyme multiplicity. The multiple forms, or isoenzymes, are glycoproteins and are denominated as B, I₁, I₂, P, A and S, in the order of decreasing isoelectric point (30 - 33). Hence, the aim of the study was to elucidate the isoenzyme pattern in those cases where total enzyme activity was elevated.

Several authors have mentioned that there are differences in the isoenzymes originating from tissues compared to those found in serum (33, 34). It has also been suggested that the serum isoenzymes are precursors of the lysosomal hydrolases (35). Isoenzymes were purified from tissue and serum with the aim to study uptake and clearance in vitro as well as in vivo. This study was planned in order to gain more information regarding the metabolism of the isoenzymes.

Finally, attempts were made to define possible clinical implications for the determination of serum β -hexosaminidase activity.

METHODOLOGICAL CONSIDERATIONS

Analytical techniques

Analysis of lysosomal enzyme activity was performed either with paranitrophenyl glycoside (Hex) or methylumbelliferyl glycoside as substrate (other lysosomal enzymes) (36).

ALAT, ALP, ASAT and GT (using L- γ -glutamyl-3-carboxy-4-nitro-anilide as substrate) were determined according to the Committee on Enzymes of the Scandinavian Society of Clinical Chemistry and Clinical Physiology (37, 38). Bilirubin was analysed using diazotized sulfanilic acid (39) or 2,4-dichloranilin (40).

Serum proteins were determined by immunoassay (41), and total serum bile acids by Stereognost[®] 3 alfa RRA (Nyegaard & Co, Oslo, Norway).

Isoelectric focusing was performed on a 110 ml column (LKB Produkter, Stockholm, Sweden) using ampholine with pH ranges of 4 - 6 or 6 - 8. The samples were electrofocused for 42 h. At the end of the experiment the voltage and current were 550 V and 0.8 mA. Two ml fractions were collected, and 100 μ l aliquots were incubated with the appropriate substrate for analysis of enzyme activity.

Purification of Hex

The purification of tissue Hex from human liver included chromatography on Con-A-Sepharose, gel filtration and ion exchange chromatography. Serum from women in their first and second trimester was used for isolation of serum Hex. The purification included chromatography using a specific affinity column. This was followed by ion exchange chromatography for isoenzyme separation and further purification. The specific activity for the various serum and tissue forms was approximately the same (varying between 800 - 3000 U/g protein) after the final purification steps.

Clearance studies

To study the clearance of Hex from rat circulation, male Wistar rats were anesthetized with 5 % chloralhydrate (0.5 ml/100 g body weight) and ether. A cannula inserted in the femoral vein was used for injection of the purified enzyme and for subsequent blood sampling. Blood samples were obtained immediately before the enzyme injection and after 2, 10, 30, 60 and 120 min. The cannula was flushed with saline after every sampling to maintain the blood volume of the rat. Heparin plasma was stored at -20°C until analysis.

Isolation of peritoneal macrophages

Mouse peritoneal macrophages from outbred female albino mice were prepared by a modification of the procedure of Cohn and Benson (42). Resident peritoneal cells were harvested in 4 ml of culture medium containing 1 % heat inactivated fetal calf serum and heparin (20 units/ml) and were plated onto plastic 6-well Linbro Tissue culture dishes. Thioglycollate stimulated cells were obtained as above 4 days after intraperitoneal injection of thio-glycollate medium. The cells were incubated at 37°C in a humidified atmosphere. Nonadherent cells were removed 2 - 3 h after plating. More than 90 % of the adherent cells were judged as viable macrophages on the basis of the following characteristics: (1) They were large, well-spread mononuclear cells which excluded trypan blue, avidly ingested latex beads ($0.81\text{ }\mu\text{m}$ diameter) and India ink (added at $10\text{ }\mu\text{l/ml}$ of medium). (2) The attachment to culture dishes was trypsin resistant ($0.025\text{ }\%$ trypsin for 2 h). (3) Numerous cytoplasmic vacuoles appeared within 30 min of exposure to concanavalin A ($0.5 - 20\text{ }\mu\text{g/ml}$ of medium). (4) Dividing cells were not observed during culture for up to 7 days plating at low density (approximately 10^5 cells/35 mm dish).

Rat peritoneal macrophages were obtained as described above, except that peritoneal cells were harvested in 15 ml of medium.

Isolation of hepatocytes and non-parenchymal liver cells

Methods described by Seglen (43) and Steer et al (44) were used with minor modifications to isolate cells from male Sprague-Dawley rats. A cell suspension was obtained by perfusion of livers with Hanks' buffer containing 40 mmol/l Hepes (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) and 0.05 % collagenase. Hepatocytes were purified by low speed centrifugation (40 x g, 1 min) at room temperature. These were suspended in culture medium containing 10 % heat inactivated fetal calf serum and antibiotics and plated ($1 - 2 \times 10^6$ cells) onto collagen coated dishes.

After removal of most of the hepatocytes by low speed centrifugation, the resultant supernatant (containing non-parenchymal liver cells) was layered over a solution of metrizamide with a density of 1.089 g/cm^3 and centrifuged (3000 x g, 45 min) at 4°C . Purified non-parenchymal liver cells consisting of Kupffer and endothelial cells were recovered by pipette from the metrizamide supernatant interface. The cells were suspended in culture medium and plated at a density of $5 - 15 \times 10^6/\text{cells per well}$. Purified Kupffer and endothelial cells could be selectively enriched by extensive washing of the non-parenchymal cell cultures. Of the still adherent cells more than 90 % showed peroxidase activity and were thus designated Kupffer cells (45). The cells removed by the washing procedures were seeded onto new dishes and consisted of approximately 80 % endothelial cells and 20 % Kupffer cells.

Uptake studies

Cells were incubated in serum-free medium containing antibiotics and various amounts of purified tissue or serum Hex were added. The medium was aspirated after 6 h and the cells were scraped from the dishes, harvested in 1 ml of distilled water and stored at -20°C until analyses. Enzyme activity and protein content of the cell homogenates were determined. Uptake was calculated as the difference in enzyme activity before and after the addition of exogenous Hex. Appropriate corrections were made for endogenous enzyme activity.

RESULTS

Conditions associated with increased Hex

Hex, ALAT, ALP, ASAT, bilirubin and GT were analysed in serum from chronic alcoholics with acute ethanol intoxication, patients with liver cirrhosis, hepatitis A, hepatitis B and cholestasis. Hex was elevated in almost all (94,4 %) chronic alcoholics with acute ethanol intoxication at the time of admission. It correlated significantly to ALAT, ASAT and bilirubin but not to GT. Decreasing values in Hex were obtained in all the patients during the observation period of seven to ten days (Paper I). Increases of Hex in cirrhosis, hepatitis A and hepatitis B correlated to ALAT and ASAT. Correlations of Hex to ALP and bilirubin were also obtained in the case of hepatitis B. Of the different enzymes, the increase of aminotransferases in hepatitis was most pronounced. Hex was increased more in cholestasis than in any other group. Despite this fact, no correlation to any "liver parameter" could be shown in cholestatic patients (Papers II and III). Hence, these findings indicate that leakage from damaged liver cells does not explain increases in serum Hex.

Infusion studies using purified human or rat tissue lysosomal enzymes have shown that the enzymes are cleared from circulation by non-parenchymal liver cells. Mannosyl/N-acetylglucosamine glycoprotein receptors on the cell surface have been implicated as being responsible for the binding and uptake of glycoproteins and lysosomal enzymes with terminal mannose and N-acetylglucosamine residues (25, 26). The non-parenchymal liver cells are part of the reticuloendothelial system (RES), and the function of this system is known to be depressed by alcohol (46) and in liver diseases (47). Hence, a feasible hypothesis at the time was that the increase in serum Hex was due to diminished clearance from circulation.

Slight to moderate increases in Hex were also observed in serum from patients with Crohn's disease and myocardial infarction. In the latter case, liver injury is often observed as a secondary reaction, probably explaining the increases in Hex. Slight increases in Hex were also seen in hyperthyroidism. This would be

expected since the thyroid hormones are known to stimulate the lysosomal apparatus (48, 49). A significant correlation between thyroid hormones and Hex has been shown in thyrotoxicosis (50).

Influence of ethanol

The finding that Hex in alcoholics was increased initiated further investigations. The activities of Hex, α -mannosidase, α -fucosidase, ASAT and GT were examined in serum from chronic alcoholics, patients with alcoholic liver cirrhosis, teetotalers and in a control group with some alcohol consumption (median 42 g alcohol per week, range 0 - 312 g). The lysosomal enzyme activities and liver enzymes were increased in both pathological groups. However, the increase of α -fucosidase in chronic alcoholics was not judged to be significant (Paper IV).

An experimental system was devised to elucidate the effect of alcohol more closely. Healthy volunteers drank 60 g of ethanol daily for 10 days. Hex, ASAT, GT, albumin, prealbumin, orosomucoid, ceruloplasmin, α_1 -antitrypsin, haptoglobin, IgA, IgG, IgM, and bile acids in serum were analysed 10 days before alcohol intake, at timed intervals during the alcohol-intake period, and after alcohol withdrawal. Hex was the only component that was altered. A small, but significant increase was observed within 3 days of ethanol consumption (first sampling occasion). This level was maintained during the period of consumption and returned to the initial levels (before alcohol intake) within 4 days of alcohol withdrawal (Paper IV). In contrast, patients with either alcoholic or non-alcoholic liver cirrhosis (51) as well as chronic alcoholics (Paper I), exhibited much higher levels of Hex, and in the case of the chronic alcoholics, the enzyme activity was elevated even on the seventh day of abstinence. Hence, these findings indicate that liver dysfunction is the main factor causing the increase of Hex. Since this dysfunction is advanced in cirrhosis and in chronic alcoholics with possible liver steatosis (52), it would explain the much higher increases in Hex, as well as a very slow return towards normality upon abstinence. An interesting feature that emerges from these studies is that serial determinations of Hex could function as one of the most sensitive parameters to monitor liver damage in patients

with alcohol related diseases.

Isoenzyme pattern of Hex

Besides the different pathological conditions mentioned, Hex and several other lysosomal hydrolases are also increased during pregnancy. The enzyme activity increases continuously during the whole period of pregnancy. No corresponding increases are found in cord blood, amniotic fluid or placental tissue (53 - 56).

Preliminary studies of Hex isoenzymes in serum from pregnant women had revealed that the increase in enzyme activity could be attributed to isoenzyme P (57). Extended studies using isoelectric focusing showed that the isoenzyme pattern of Hex was abnormal, but similar, in serum from women in the second and third trimester, patients with liver cirrhosis, cholestasis and chronic alcoholics with acute ethanol intoxication. A preferential increase of Hex P (pI between 5.6 and 6.8) was evident in each case (Paper V).

The lysosomal enzymes Hex, α -fucosidase, β -glucuronidase, α -hexosaminidase and α -mannosidase were examined in serum of pregnant women, approaching term, and at intervals during the first six days after parturition. The multiple forms of the different enzymes were also investigated. All the enzyme activities had increased at term, and all activities except for α -fucosidase had returned to normal levels during the 6 days after parturition, but at different rates. The data on isoenzymes was informative only in the case of Hex and showed that pre- and post-partum changes in Hex depended largely on changes in the activity of the P form (Paper VI). Since the same isoenzyme was also increased in liver diseases, it was assumed that fluctuations in Hex levels (as for instance after alcohol withdrawal) might well be due to Hex P. It is likely that there is a common underlying mechanism responsible for changes in this isoenzyme in these cases.

Uptake and clearance of Hex

Uptake studies of Hex were conducted by adding either isoenzyme A or B, purified from human liver, to different cell cultures containing non-parenchymal cells from rat liver, peritoneal macrophages from mouse or the same cells from rat. The activities of Hex and several other lysosomal hydrolases were determined in cell homogenates and culture medium obtained before and after the addition of Hex. Uptake was calculated as the difference in these activities after correction for endogenous Hex.

A considerable uptake of each isoenzyme of Hex was observed in non-parenchymal liver cells but not in the peritoneal macrophages. Thus, it seemed that there was at least a quantitative difference in the receptor mechanism of liver and peritoneal macrophages (Paper VII). Studies on the subpopulations of the non-parenchymal liver cells showed that endothelial cells were capable of accumulating between 3 and 4 times as much Hex as the Kupffer cells. The uptake exhibited saturation kinetics and could be inhibited competitively by mannan, which is a potent inhibitor of cellular binding when glycoproteins with either terminal mannose or N-acetylglucosamine residues are involved. These findings supported the concept that a cell surface receptor in non-parenchymal liver cells mediated the uptake of Hex.

The results for Hex uptake with non-parenchymal liver cells were in agreement with those obtained by others, using either a similar technique (58) or radio-iodine labelled Hex as ligand (44). However, different findings have been reported for macrophages. For instance, Stahl et al have demonstrated the binding of β -glucuronidase to rat alveolar macrophages (59) and Kusiak et al have reported the binding of Hex A to cultured rat peritoneal macrophages (60). In both cases, radio-iodinated ligands were used. It is possible that methodological differences in the preparation of ligands and/or in detection techniques contribute to these discrepancies (61).

The clearance of tissue lysosomal enzymes from circulation in the rat occurs mainly in the liver. Infused lysosomal enzymes or glycoproteins with terminal mannose or N-acetylglucosamine residues are cleared slowly in hepatectomized rats and show a diffuse distribution with some accumulation in bone and spleen (24). Electrone microscope autoradiography of the rat liver have shown that the endothelial cells were most efficient in clearing β -glucuronidase and glycoproteins with terminal mannose or N-acetylglucosamine residues (27). These findings are consistent with the results that we have obtained in vitro (Paper VII).

The uptake of purified serum Hex isoenzymes A, B and P was also studied in rat liver non-parenchymal cells and hepatocytes. In contrast to tissue Hex, no detectable uptake of any serum isoenzyme was noticed in either cell type. However, when these isoenzymes were desialylated by neuraminidase treatment, isoenzymes A and B were taken up by the non-parenchymal cells only. No uptake was observed for Hex P. None of the desialylated serum forms were taken up by hepatocytes (Paper VIII).

Infusion of human liver Hex A and B in rats resulted in their elimination from circulation within 10 min, whereas the elimination of serum Hex A, B or P was slower. This elimination was enhanced when desialylated isoenzymes were infused (Paper VIII). These results are consistent with those reported by Bearpark and Stirling (62).

GENERAL DISCUSSION

The initial part of this thesis deals with several diseases where the activities of lysosomal enzymes are increased. Particular attention was paid to Hex since the activity of this enzyme usually showed the most significant changes in the diseases studied. Serum Hex in patients with liver cirrhosis, hepatitis, cholestasis and alcoholism was related to known "liver parameters" in order to gain better insight on the underlying mechanism(s) responsible for the observed increases of the lysosomal enzyme activity. The correlation studies in most of these diseases could indicate that leakage of Hex from damaged liver cells occurred. However, failure to attain any such correlation in cholestasis (despite the fact that Hex showed the largest increases in this group) indicated that liver dysfunction rather than leakage was the primary reason for increased Hex in these diseases.

Conditions where increases in Hex could not be attributed to liver dysfunction included diabetes mellitus, thyrotoxicosis and pregnancy. A slight to moderate increase in Hex, correlating closely to blood glucose and glycosylated hemoglobin ($\text{HbA}_{1\text{C}}$) was observed in diabetes mellitus (63 - 66). In vitro studies with human fibroblasts grown in a glucose-enriched medium showed that the release of Hex correlated to the glucose concentration in the medium (67). Hence, it is reasonable to assume that high concentration of extracellular glucose stimulates the release of lysosomal enzymes, including Hex, in vivo. In hyperthyroidism, the increased serum levels of Hex seem related to the well-known stimulation of the lysosomal apparatus by thyroid hormones (48). The reason for the increase of Hex in pregnancy is not clear, although both liver and placental involvement have been implicated (53, 68). It is possible that steroid hormones function as stimuli (68). This suggestion is supported by the finding that there is a relationship between Hex and the steroid dose in women on contraceptives (69).

As regards the isoenzyme pattern, Hex P is normally a minor component and is practically unchanged in either diabetes mellitus or thyrotoxicosis (66, 50). However, it was a dominant fraction during pregnancy and fluctuations of this component were

mainly responsible for the changes in total Hex either during pregnancy or post partum. Since increased Hex P was also seen in serum from chronic alcoholics and patients with liver diseases, it was suggested that a common mechanism was operative in these situations.

Macrophages seem to play a major role in the secretion of lysosomal enzymes (11). A variety of endogenous and exogenous compounds, such as endotoxins and immune complexes are known to stimulate the macrophages (19 - 21, 70). Plasma levels of these agents are elevated in liver diseases because the function of Kupffer cells, which clear them from the circulation, is depressed (71). Likewise, endotoxemia has been reported in a large number of patients with obstructive jaundice (72 - 74). The involvement of the macrophage in secretion is further indicated by the observation that increased activities of serum β -glucuronidase and Hex in experimental cholestasis and cirrhosis in rats were associated with the accumulation of macrophages in liver and spleen (75, 76). Increased amounts of macrophages were also found in the lungs of rats with cholestasis (77). In liver disease, the total body macrophages may be activated by enteric antigens bypassing the filtering action of the liver. This concept is supported by the finding that Hex is elevated in the monocytes of patients with chronic liver disease (78). Besides the activated macrophages, other cell types such as hepatocytes (75) and fibroblasts (79) may also contribute to increased Hex in liver diseases. Activation of macrophages by estrogens (80) may be responsible for the rise in Hex found during pregnancy. However, the contribution from hepatocytes may also be relevant in this case, since estrogens also induce synthesis of several liver derived plasma proteins.

Activated monocytes are thought to be responsible for the elevated serum Hex found in inflammatory bowel diseases (Crohn's disease and ulcerative colitis) (81). Some infections are also known to activate macrophages. For instance, injection of BCG (an attenuated strain of Tuberculin bacilli) to mice results in elevated Hex in plasma, liver and peritoneal macrophages (82). Increased Hex was also found in macrophages from rats injected with Corynebacterium parvum (83). In fact, Schrecker et al

(working with immuno-adjuvants in mice) have suggested that serum activities of lysosomal enzymes could serve as biochemical markers of macrophage activation (84).

All these observations indicate that the activated macrophage plays an important role in the secretion of the lysosomal enzymes. The nature of the enzyme secreted depends on the type of stimulant as well as the duration of stimulation. For instance, zymosan stimulation during a short period (1 - 4 h) resulted in the secretion of mature lysosomal enzymes (tissue forms). However, prolonged exposure to the same agent resulted mainly in the secretion of precursors (20, 21). A preferential secretion of the precursors can also be achieved by short term exposure to agents favouring diminished recycling of Man-6-P receptors. The precursor forms have been suggested to be the origin of the enzyme forms found in serum (35).

Experimental evidence has been presented to show that tissue forms of Hex are cleared from rat circulation more rapidly than the serum forms. Since similar clearance patterns of the lysosomal enzymes have been observed in rat and man (85 - 87), it can be assumed that the underlying mechanisms responsible for clearance are similar in each case. There is evidence showing that desialylation of serum isoenzymes enhances their clearance rate by the non-parenchymal liver cells in rat. However, it is uncertain if desialylation occurs in vivo and whether this is a prerequisite for the normal turnover of sialylated serum glycoproteins (88).

Regarding uptake, it is known that the terminal residue of most desialylated glycoproteins is galactose (89). Hence, one would have expected that the galactose receptors on hepatocytes would be involved in their uptake. This hypothesis could not be substantiated. Instead, non-parenchymal liver cells were found to be involved in the uptake of desialylated Hex A and B purified from serum. It is possible that a galactose receptor is involved, since some investigators claim the existence of these receptors on non-parenchymal liver cells (90, 91). Alternatively, it may be possible that removal of sialic acid makes the isoenzymes recognizable via mannose-rich oligosaccharide chain(s), which might

co-exist together with the complex oligosaccharide chains as in glucocerebrosidase and α -mannosidase (92, 93). A third alternative is that uptake is mediated via a receptor that has not yet been characterized. Uptake studies with Hex P were negative. This was surprising in view of the fact that Hex P has been suggested to contain more sialic acid than Hex B (94). Since asialo Hex P is cleared rapidly from circulation when injected, it is likely that another cell type is responsible for its clearance.

CLINICAL IMPLICATIONS

Increased serum Hex in chronic alcoholics is probably due to alcohol-induced liver dysfunction rather than to a direct effect of the alcohol. However, serial determinations of Hex might be useful to monitor patients with alcohol related diseases, since a major change in its activity strengthens the suspicion of liver damage.

Endotoxemia has been reported in a large proportion of patients with liver disease (95, 96). As yet, it is not clear if there is a correlation between serum Hex and endotoxemia. This is worthwhile studying because there is growing evidence of the prognostic value of endotoxemia in liver diseases. Since endotoxemia is technically difficult to assess, the determination of Hex may provide a useful alternative.

Further studies on Hex isoenzymes and their metabolism using monoclonal antibodies may help to solve the question of pathophysiological impact of serum isoenzymes. Likewise, the question of an underlying mechanism, probably responsible for the similar enzyme pattern in alcoholism, liver diseases and pregnancy needs to be examined.

SUMMARY

- 1 The present study has shown or confirmed that serum/plasma Hex is increased in pregnancy, chronic alcoholism and liver disease.
- 2 The isoenzyme pattern of serum Hex shows a preferential increase of the isoenzyme designated Hex P in all the above mentioned conditions indicating a common mechanism for the increase.
- 3 Native serum Hex isoenzymes are cleared more slowly from rat circulation than tissue forms. The clearance of the serum forms is enhanced after desialylation.
- 4 None of the native forms of Hex in serum are taken up by hepatocytes or non-parenchymal liver cells in vitro. Of the desialylated isoenzymes, only Hex A and B are taken up by the non-parenchymal liver cells.
- 5 Tissue Hex A and B are taken up by the non-parenchymal liver cells but not by peritoneal macrophages in vitro, indicating a difference in the receptor function on liver and peritoneal macrophages.
- 6 Monitoring of serum Hex could function as a sensitive variable reflecting alcohol ingestion.

Serum Hex may also be useful to analyze in patients with alcohol related diseases, where major changes in enzyme activity could give rise to a suspicion of liver injury.

In liver disease, analysis of serum Hex may give additional information such as reflecting the degree of endotoxemia.

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β -HEXOSAMINIDASE, LEUCINE AMINOPEPTIDASE, CYSTIDYL AMINOPEPTIDASE, HEPATIC ENZYMES AND BILIRUBIN IN SERUM OF CHRONIC ALCOHOLICS WITH ACUTE ETHANOL INTOXICATION

B. HULTBERG *, A. ISAKSSON and G. TIDERSTRÖM

Department of Clinical Chemistry, Central Hospital, S-351 85 Växjö (Sweden) and University Hospital, S-221 85 Lund (Sweden)

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Summary

β -Hexosaminidase, leucine aminopeptidase, cystidyl aminopeptidase and routine liver parameters are studied in serum of chronic alcoholics with acute ethanol intoxication.

The frequencies of pathological serum levels of the routine measurements made are in relatively good agreement with earlier reports.

Serum leucine aminopeptidase and cystidyl aminopeptidase did not show any significant aberration in alcoholic liver disease. They correlated well with each other and γ -glutamyltransferase.

β -Hexosaminidase activity in serum was increased in almost all patients at the time of admission. All the patients showed decreasing values during the observation period.

Introduction

Many lysosomal glycosidases (and other glycoproteins) are cleared from plasma by a specific recognition system largely associated with the liver [1]. After uptake the enzymes are rapidly transported to the liver lysosomes. It has been shown that β -hexosaminidase (EC 3.2.1.30) is preferentially taken up by the non-parenchymal cells of (rat) liver [1].

In an earlier investigation [2], we found increased values of acid hydrolases in lysosomal storage disorders and attributed those findings to the hepatopathy which often occurs in the lysosomal diseases because of the accumulated material stored in the lysosomes.

* Correspondence should be addressed to B. Hultberg, University of Lund, Department of Clinical Chemistry, University Hospital, S-221 85 Lund, Sweden.

The present paper evaluates the serum activities of β -hexosaminidase (a lysosomal hydrolase) in patients with ethanol-induced liver cell dysfunction. The toxic effects of ethanol on the liver are well known [3]. However, the mechanism by which ethanol damages the liver and the factors determining the susceptibility of any individual to liver damage are unknown.

Cystidyl aminopeptidase (CAP) (EC 3.4.11.3) and leucine aminopeptidase (LAP) (EC 3.4.11.1) were also evaluated in these patients with alcoholic liver diseases, since an earlier paper [4] suggested that analysis of both aminopeptidases might give some information about the origin of aminopeptidase activities in serum. For the same reason both CAP and LAP were analysed with and without the addition of Co^{2+} (0.1 mmol/l) to the reaction mixture.

Material and methods

The patients consisted of 47 males and 7 females, aged 28–70 years (47 ± 10 , mean $\pm$ standard deviation). The patients had been hospitalized for detoxication because of acute ethanol intoxication. All fulfilled the criteria of γ -alcoholism according to the classification of Jellinek [5,6]. None had a diagnosis of liver cirrhosis. The protein pattern was determined in all patients on arrival (albumin, orosomucoid, α_1 -antitrypsin, haptoglobin, ceruloplasmin, immunoglobulin A, G and M). The results of this investigation suggested a well preserved capacity for protein synthesis in all patients. A slight inflammatory reaction was seen in about 50% of the patients. In 10% of the patients immunoglobulin A was increased.

The statistical calculations were made on analysis of blood samples drawn the day after admission and after the 7–10th day of hospitalization.

The reference group for β -hexosaminidase, CAP, and LAP comprised 29 controls (20 males and 9 females) aged 24–60 years.

Bilirubin (reference range 3–20 $\mu\text{mol/l}$) was determined by the method of Nosslin [7]. The serum activities of aspartate aminotransferase (ASAT) ($<0.7 \mu\text{kat/l}$), alanine aminotransferase (ALAT) ($<0.7 \mu\text{kat/l}$), and γ -glutamyltransferase (GT) ($<1.0 \mu\text{kat/l}$) were determined with a Vitatron AKES enzyme analyzer using the methods of the Committee of Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology [8]. Activities of β -hexosaminidase were assayed as described previously [2].

CAP and LAP activities were assayed as described elsewhere [4,9], except that both methods were adapted for use on the Vitatron AKES enzyme analyzer without any alteration in the reaction conditions.

For statistical evaluation of the results, means and standard deviations were calculated. When comparing different groups of patients Student's *t* test was used. The different analyses were compared to each other with linear regression analysis and the *r* values were tested for significance with the *t* test.

Results

On arrival at the hospital 56.6% of the patients had an elevated concentration of bilirubin ($23.4 \pm 9.8 \mu\text{mol/l}$; mean $\pm$ standard deviation). At the end of the observation period (7–10 days) serum bilirubin was normal in all patients ($8.4 \pm 4.2 \mu\text{mol/l}$).

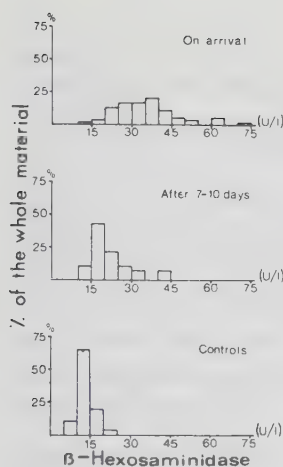


Fig. 1. β -Hexosaminidase in controls and patients on arrival and after 7–10 days of abstinence. Activity given as U (= μ mol substrate split per min) per litre serum.

On admission, 81.5 and 58.5% of the patients had increased activities of serum ASAT ($1.79 \pm 1.38 \mu\text{kat/l}$) and serum ALAT ($1.35 \pm 1.18 \mu\text{kat/l}$), respectively. At the end of observation 35.7 and 51.8% of the patients still had abnormally high serum ASAT ($0.75 \pm 0.40 \mu\text{kat/l}$) and serum ALAT ($1.11 \pm 0.93 \mu\text{kat/l}$) activity.

Initially 66% of the patients had an increased activity of serum GT ($2.60 \pm 3.18 \mu\text{kat/l}$). After 7–10 days of abstinence 70% of all patients had an increased serum GT ($2.05 \pm 1.37 \mu\text{kat/l}$).

β -Hexosaminidase activity was increased in 94.4% of the patients on admission and after 7–10 days 46.4% of the patients still showed an elevation. During the observation period all patients showed a decrease of β -hexosaminidase activity (Fig. 1). Mean and standard deviation of controls and patients on arrival and after 7–10 days of abstinence are given in Table I.

Serum LAP activity showed a normal distribution both on admission and after 7–10 days compared to the controls. Only 13% of the patients had

TABLE I

β -HEXOSAMINIDASE IN CONTROLS AND PATIENTS ON ARRIVAL AND AFTER 7–10 DAYS OF ABSTINENCE

(Mean and standard deviation are given. Activity given as U (= μ mol substrate split per min) per litre serum)

	Controls	Patients at arrival	Patients after 7–10 days of abstinence
β -Hexosaminidase	13.5 ± 3.5	35.4 ± 12.4	21.7 ± 8.1

TABLE II

LAP AND CAP IN CONTROLS AND PATIENTS ON ARRIVAL AND AFTER 7-10 DAYS OF ABSTINENCE

(Mean and standard deviation are given. Activity given as $\mu\text{kat/l}$ serum. Both enzymes are assayed with and without Co^{2+} -ions)

	LAP	LAP Co	CAP	CAP Co
Controls	1.75 ± 0.54	1.47 ± 0.51	0.08 ± 0.04	0.12 ± 0.04
Patients on admission	1.62 ± 0.64	1.54 ± 0.73	0.10 ± 0.05	0.16 ± 0.05
Patients after 7-10 days of abstinence	1.52 ± 0.58	1.51 ± 0.61	0.13 ± 0.05	0.18 ± 0.07

increased serum CAP value at the time of admission. Mean and standard deviation are given in Table II. Serum LAP and serum CAP are assayed with and without Co^{2+} -ions.

In Table III the correlation coefficient, t value and degree of significance are given for the analyses performed on patients on arrival. Only statistically significant correlations are included in the table ($p < 0.05$).

A highly significant correlation was found between the serum activities of ASAT and ALAT. Serum ASAT, ALAT and GT showed a slightly significant correlation towards serum bilirubin.

β -Hexosaminidase correlated best with serum ASAT and ALAT, but also with serum bilirubin. There was no correlation with serum GT.

Serum LAP (Co^{2+} -stimulated) correlated significantly with serum LAP, GT, CAP (Co^{2+} -stimulated) and CAP. There was no correlation with β -hexosami-

TABLE III

CORRELATION COEFFICIENTS (r), NUMBER OF PAIRS (n), t -VALUES (t) AND DEGREE OF SIGNIFICANCE (p) OF THE VARIOUS TESTS PERFORMED ON THE PATIENTS ON ADMISSION

Tests	r	n	t	p
ASAT/ALAT	0.88	53	13.4	<0.001
ASAT/Bilirubin	0.22	53	1.6	<0.05
ALAT/Bilirubin	0.22	52	1.6	<0.05
GT/Bilirubin	0.25	52	1.8	<0.05
β -Hexosaminidase/ASAT	0.53	54	4.5	<0.001
/ALAT	0.48	53	4.0	<0.001
/Bilirubin	0.34	53	2.6	<0.02
LAPCo/LAP	0.91	43	13.8	<0.001
/GT	0.68	43	6.0	<0.001
/CAPCo	0.69	39	5.7	<0.001
/CAP	0.67	33	5.0	<0.001
LAP/GT	0.53	44	4.0	<0.001
/CAPCo	0.53	40	3.9	<0.001
/CAP	0.54	34	3.6	<0.001
CAPCo/CAP	0.84	34	8.9	<0.001
/GT	0.69	40	5.9	<0.001
CAP/GT	0.69	34	5.5	<0.001

TABLE IV

β -HEXOSAMINIDASE ACTIVITY IN DIFFERENT GROUPS OF PATIENTS (WITHOUT LIVER AFFECTION)

(Mean and standard deviation are given)

Patients	β -Hexosaminidase
Normal protein pattern (plasma) (n = 100)	12.3 $\pm$ 3.6
Slight non-specific activity (plasma) (n = 50)	13.4 $\pm$ 2.8
Moderate non-specific activity (plasma) (n = 50)	13.8 $\pm$ 2.5
High non-specific activity (plasma) (n = 50)	13.0 $\pm$ 3.7
Haptoglobin consumption (plasma) (n = 10)	11.4 $\pm$ 5.4
Hyperthyroidism (serum) (n = 12)	24.7 $\pm$ 8.7 *
Hypothyroidism (serum) (n = 13)	14.1 $\pm$ 2.8
Rheumatoid arthritis (serum) (n = 30)	14.8 $\pm$ 4.2
Crohn's disease (serum) (n = 30)	17.9 $\pm$ 4.6 *
Myocardial infarction (serum) (n = 40)	23.9 $\pm$ 7.12 *

* $p < 0.05$.

dase, serum bilirubin or the transaminases. Serum LAP showed the same pattern.

Serum CAP (Co^{2+} -stimulated) correlated very well with serum CAP and GT while serum bilirubin, β -hexosaminidase, serum ALAT and ASAT showed no correlation with CAP (Co^{2+} -stimulated).

In order to evaluate further the possible role of β -hexosaminidase in the diagnosis of liver disease, different groups of patients with other diseases were studied (Table IV).

EDTA-plasma intended for the investigation of the protein pattern from 260 patients with different diseases (liver affections were not included) were also analyzed for β -hexosaminidase activity. 100 patients had a normal protein pattern. Groups of 50 patients were collected with slight non-specific activity (serum orosomucoid corresponding to 1.10–1.80 g/l; control value 0.50–1.05), moderate non-specific activity (orosomucoid 1.80–2.70 g/l) and high non-specific activity (orosomucoid >2.70 g/l). In 10 patients there was consumption of haptoglobin because of hemolytic disease and these patients are represented as a separate group in Table IV. As can be seen in Table IV none of these groups exhibited any significant difference from the controls.

Some other groups of patients were also studied and they are presented in Table IV. All these patients exhibited typical clinical, biochemical and/or histopathological findings for the disease in question. The patients were in the same age group as the controls, except for patients with myocardial infarction who exhibited a higher age distribution (range 50–82). Patients with hyperthy-

roidism, myocardial infarction and Crohn's disease exhibited a significantly higher mean value than controls.

Discussion

None of the patients included in these investigations had any clinical symptoms of liver cirrhosis. All of them exhibited a well-preserved capacity for protein synthesis.

The frequencies of pathological serum levels of the routine parameters studied are in relatively good agreement with earlier reports [10–12].

The only aberrant finding in this investigation is a higher frequency of increased serum bilirubin compared to earlier studies [10,12]. We have no explanation for this discrepancy. The increase of serum bilirubin has been attributed not only to liver damage but also to the dietary deficiencies often occurring in these patients [12]. During the first week after admission a rapid decrease was seen, even to subnormal values. This has been attributed to a diminished destruction of erythrocytes and/or increased activity of glucuronyl transferase caused by the ethanol enzyme induction [12].

Serum LAP and CAP did not show any significant aberration in alcoholic liver disease. They correlated well with each other and GT. LAP is well known as a membrane enzyme [13] and the significant correlation with GT is therefore not unexpected. Co^{2+} -stimulation did not give any further information.

β -Hexosaminidase activity in serum was increased in almost all patients at the time of admission. All the patients showed decreasing values during the observation period. In almost all patients the same finding was seen for serum ASAT and bilirubin. GT, ALAT, CAP and LAP showed a more complex pattern with a slight to moderate decrease, or unchanged level or even a slight increase.

β -Hexosaminidase correlated with the measurements that indicated membrane leakage or cell death (ASAT, ALAT and bilirubin). It was not correlated with GT, which is considered to be a very sensitive indication of ethanol-induced liver dysfunction [10,14]. The rise of GT activity is dependent on stasis in the bile capillaries. The highly significant correlation with β -hexosaminidase towards "cell death" indicators does not disprove the hypothesis proposed in the introduction, because dysfunction or death of liver cells must also result in a defective clearance of β -hexosaminidase from the serum. Further support for this hypothesis is the known fact that ethanol is a drug which depresses the function of the reticuloendothelial system in the liver [15].

Acid hydrolases have been studied very little in alcoholic liver disease, but in one earlier investigation [16] significant elevations were found for arylsulfatase and β -glucuronidase in the plasma of alcoholics with acute alcoholic intoxication.

Seymour and Peters [13] used enzyme analysis coupled with analytical sub-cellular fractionation of liver tissue to explore the possible pathogenesis of alcoholic liver disease.

Apart from a small decrease in activity of certain acid hydrolases in liver steatosis, the activity of lysosomal hydrolases was unaffected by alcoholic liver disease [13]. Similarly, measurements of lysosomal integrity did not show any

significant alteration. This finding implies that lysosomal disruption is not responsible for the increased β -hexosaminidase found in serum.

Further evaluation of β -hexosaminidase in different pathological conditions shows that the degree of activity (as measured by protein pattern) does not influence the level of the enzyme in serum. High mean activity in hyperthyroidism probably depends upon the stimulation effect that thyroxine exerts upon the lysosomal system [17]. The reason for the increase of the mean value in Crohn's disease and myocardial infarction might depend upon the known hepatic involvement in these conditions. The increase found in myocardial infarction might also partly derive from the higher age group. β -Hexosaminidase is known to increase slightly with age [18].

In conclusion, these results suggest that β -hexosaminidase levels (or "endogenous clearance" of β -hexosaminidase) might be a useful measure of liver cell function, mainly reflecting the function of the non-parenchymal cells because they are thought to be responsible for the clearance of β -hexosaminidase from the plasma [1,19].

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β -Hexosaminidase in Serum from Patients with Cirrhosis and Cholestasis

Björn Hultberg, Anders Isaksson, Leif Jansson

Department of Clinical Chemistry, University Hospital, Lund, and Department of Clinical Chemistry,
Central Hospital, Växjö, Sweden

Key Words. Cholestasis · Cirrhosis · β -Hexosaminidase · Serum

Abstract. The enzyme activity level of β -hexosaminidase (β -N-acetylglucosaminidase, EC 3.2.1.30; 2-acetamido-2-deoxy- β -D-glucoside acetamidodeoxyglucohydrolase) in serum from patients with different forms of cirrhosis and cholestasis was found to be elevated. A comparison is also made with routine liver parameters. In the light of recent findings it seems probable that the increased level depends upon depressed clearance of this enzyme by the non-parenchymal cells in the liver.

Introduction

Our understanding of the specific functions of sinusoidal (non-parenchymal) cells of the liver is limited compared to what we know about parenchymal cells (hepatocytes). Indirect data have been collected by studying the reticulo-endothelial system (RES) clearance under normal and many experimental conditions [1].

The function of the RES in human liver disease is apparently ill defined and further evaluation is mandatory [2]. The RES is incriminated as the main clearance organ. Most of the RES (80–90%) is represented by the Kupffer cells of the liver. Diseases of the liver may interfere with their clearance properties.

The few data available indicate preferentially suppressed RES function, especially in cirrhosis [3], probably due to hemodynamic disturbances. Age, hormones, and stress also play a role [2]. Viruses as well as toxic agents, particularly alcohol, may decrease the RES function [2–4]. Thus, in liver disease, the clearance system for endotoxins is insufficient to eliminate them from the bloodstream when they enter the general and/or portal circulation. The pathogenetic significance of endotoxins in various liver diseases has been discussed [2].

Most techniques used in man to assess the function of RES measure the rate of clearance of small immunologically inert particles [5]. More recent methods for measuring clear-

ance are more immunologically specific because they depend on receptors for C3b and IgG [6, 7].

Recent evidence suggests that some glycoproteins, e.g. β -hexosaminidase (a lysosomal enzyme) are cleared from the bloodstream by means of specific receptors localized on the cell membrane of the non-parenchymal cells in the liver [8, 9]. In an earlier investigation we showed that acute alcohol intoxication increased the level of β -hexosaminidase in serum [10]. We attributed the phenomenon to depression of the RES.

We have therefore continued to study the 'endogenous clearance' of β -hexosaminidase in other liver diseases in order to shed light upon the involvement of non-parenchymal cells (RES) in these diseases.

Materials and Methods

The reference group for β -hexosaminidase comprised 29 controls (20 males and 9 females) aged 24–60 years.

The diagnosis of patients with hepatobiliary diseases was based on the presence of typical clinical, serum biochemical, hepato-histologic and, where appropriate, cholangiographic findings. All patients' sera were negative for hepatitis B surface antigen.

Primary Biliary Cirrhosis (PBC) ($n = 10$). Interpretation of liver biopsies and the determination of histological stages of PBC were according to the criteria of Scheur [11]. The patients with PBC varied widely in terms of duration of symptoms, degree of cholestasis and histological stage of the disease.

Other Forms of Cirrhosis ($n = 45$). This group consisted of 20 patients with alcoholic cirrhosis, 15 patients with cirrhosis caused by hepatitis, and 10 other patients with cryptogenic cirrhosis. In addition to the above findings all patients also gave a protein pattern typical for cirrhosis [12].

Patients with Cholestasis ($n = 16$). 5 patients had a severe form of choledocholithiasis and 6 had cholestasis secondary to malignant processes in the biliary duct or pancreas. 3 patients had atresia of the biliary ducts and the other 2 intrahepatic cholestasis due to metastasis of breast carcinoma. All patients had had their cholestasis for more than 1 week prior to sampling.

Bilirubin (reference range 3–20 $\mu\text{mol/l}$), aspartate aminotransferase (ASAT) ($< 0.7 \mu\text{kat/l}$), alanine aminotransferase (ALAT) ($< 0.7 \mu\text{kat/l}$), alkaline phosphatase (ALP) ($< 4.7 \mu\text{kat/l}$), and γ -glutamyltransferase (GT) ($< 1.0 \mu\text{kat/l}$) were determined as described earlier [10].

β -Hexosaminidase (EC 3.2.1.30) assay was performed as follows: 50 μl *p*-nitrophenyl-2-acetamido-deoxy- β -D-glucopyranoside (Koch-Light Labs. Ltd., Colnbrook, England), 7.5 mol/l in water, and 50 μl 1 mol/l citrate buffer, pH 4.0. and 100 μl serum were incubated at 37 °C for 30 min. The reaction was stopped with 3 ml of 0.2 mol/l glycine buffer, pH 10.7, and read within 30 min in a spectrophotometer at 400 nm. *p*-Nitrophenol was used as the standard. The amount of enzyme is defined as micromoles of substrate split per minute (U) per liter serum.

For statistical evaluation of the results, means and standard deviations were calculated. The different analyses were compared to each other with linear regression analysis and the coefficient correlations were tested for significance with the Student's *t* test. Only $p < 0.05$ is regarded as significant.

Results

In figure 1 a clear increase in the β -hexosaminidase level in serum from patients with cirrhosis is seen. Results from cryptogenic and posthepatic cirrhosis are calculated together with the results from cirrhosis caused by ethanol since there were no discrepancies between the different cirrhotoses when calculated in separate groups. In table I the mean $\pm$ standard deviation are given for the different liver tests and β -hexosaminidase. The range for β -hexosaminidase was 11–42 U/l. A significant correlation was obtained between β -hexosaminidase and the aminotransfer-

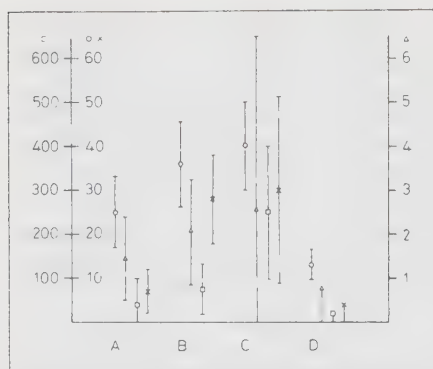


Fig. 1. Mean $\pm$ standard deviation for β -hexosaminidase ($\circ$), S-ASAT (Δ), S-Bilirubin ($\square$), and S-ALP ($\times$). **A** Cirrhosis. **B** Primary biliary cirrhosis. **C** Cholestasis. **D** Controls (in controls only the upper reference limits are given for S-ASAT, S-bilirubin, and S-ALP).

Table I. Serum findings in cirrhosis ($n = 45$)

	Mean $\pm$ SD	
β -Hexosaminidase	24.9 ± 8.9	
Bilirubin	39.0 ± 56.5	
ALP	6.7 ± 5.1	
GT	3.1 ± 2.5	
ASAT	1.4 ± 0.9	
ALAT	0.8 ± 0.6	
	Correlation coefficient	Significance
β -Hexosaminidase/bilirubin	0.01	n.s.
β -Hexosaminidase/ALP	0.09	n.s.
β -Hexosaminidase/GT	0.15	n.s.
β -Hexosaminidase/ASAT	0.51	$p < 0.001$
β -Hexosaminidase/ALAT	0.37 *	$p < 0.02$

ases. β -Hexosaminidase showed no correlation with the other routine parameters.

Correlation between the different routine liver parameters was significant between ASAT/ALAT ($p < 0.001$) and between ALP/GT ($p < 0.05$).

Results from patients with primary biliary cirrhosis were calculated separately from the other cirrhotics because of the cholestatic feature in this disease. β -Hexosaminidase was also increased in these patients, but no correlation with the other routine liver parameters was noted (table II). The range for β -hexosaminidase was 18.9–52.0 U/l.

In order to elucidate the findings in primary biliary cirrhosis, patients with severe cholestasis caused by various diseases were investigated. Even in these disorders β -

Table II. Serum findings in primary biliary cirrhosis ($n = 10$)

	Mean $\pm$ SD	
β -Hexosaminidase	36.6 ± 9.5	
Bilirubin	73.1 ± 56.2	
ALP	28.2 ± 10.3	
GT	16.3 ± 11.4	
ASAT	2.1 ± 1.2	
ALAT	1.2 ± 0.8	
	Correlation coefficient	Significance
β -Hexosaminidase/bilirubin	0.14	n.s.
β -Hexosaminidase/ALP	0.02	n.s.
β -Hexosaminidase/GT	0.48	n.s.
β -Hexosaminidase/ASAT	0.28	n.s.
β -Hexosaminidase/ALAT	0.11	n.s.

hexosaminidase was increased (range 23–65) and showed no correlation to the different routine tests (table III).

Discussion

Non-parenchymal cells have been shown to exhibit structural alteration in many different liver diseases, e.g. both in cirrhosis and cholestasis [13]. The dysfunction of RES in cirrhosis is also supported by the fact that 79% of patients with cirrhosis showed endotoxemia [2].

In an earlier study [14] increase in β -hexosaminidase was noted in sera from patients with cirrhosis. Pott et al. [14] considered the increased level to depend upon the

activity of mesenchyme reaction. In light of recent results (see 'Introduction') it seems probable that increased β -hexosaminidase levels depend on depressed clearance of this enzyme by the non-parenchymal cells in the liver.

In this investigation the only correlation that was noted between β -hexosaminidase and the other liver tests was between β -hexosaminidase and the aminotransferases in the first group of cirrhotics. The level of the aminotransferases is thought to express the degree of activity of the cirrhosis. Thus a cirrhosis at a high activity stage in the disease also shows a profound influence on the non-parenchymal cells in the liver. However, patients with almost normal liver tests could exhibit very high levels of β -hexosaminidase. One explanation of these findings could be that in cirrhosis the non-parenchymal cells cannot display their optimum clearance properties since they have lost contact with much of the portal blood [15]. The differences in the amount of portocaval collateral circulation between one patient with cirrhosis and another may therefore explain different levels of β -hexosaminidase activity in serum.

In serum from patients with primary biliary cirrhosis and cholestasis there was no correlation of β -hexosaminidase with the other routine liver tests. Increased levels of β -hexosaminidase were seen in both groups.

In an earlier study patients with primary biliary cirrhosis had a defect in clearance mediated by C3b receptors [6]. No such defect was found in patients with other forms of cirrhosis or cholestasis. The defect was specific since clearance of IgG-sensitized autologous erythrocytes or microaggregated albumin was normal or even accelerated. One possible explanation for this finding would be

Table III. Serum findings in cholestasis (n = 16)

	Mean $\pm$ SD	
β -Hexosaminidase	40.3 $\pm$ 10.6	
Bilirubin	251.2 $\pm$ 156.2	
ALP	30.3 $\pm$ 22.3	
GT	11.8 $\pm$ 10.6	
ASAT	2.6 $\pm$ 3.6	
ALAT	1.8 $\pm$ 1.8	
	Correlation coefficient	Significance
β -Hexosaminidase/ bilirubin	-0.04	n.s.
β -Hexosaminidase/ ALP	-0.23	n.s.
β -Hexosaminidase/ GT	-0.13	n.s.
β -Hexosaminidase/ ASAT	-0.01	n.s.
β -Hexosaminidase/ ALAT	-0.08	n.s.

a decreased availability of these receptors in PBC. This could occur if a large proportion of them are occupied by either excess free C3b or immune complexes containing C3b.

Future investigations of hepatic cell surface receptors will provide valuable new insight into hepatic physiology and pathophysiology as well as mechanisms of liver injury. The clearance mechanism of β -hexosaminidase will therefore be studied further in a large group of cirrhotic patients which will be followed for several years. It should then be possible to correlate β -hexosaminidase level to duration, severity and progress of the cirrhosis.

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Björn Hultberg, Department of Clinical Chemistry, University Hospital, S-221 85 Lund (Sweden)

β -Hexosaminidase Level in Serum from Patients with Viral Hepatitis as a Measure of Reticuloendothelial Function

BJÖRN HULTBERG,¹ JEAN HENRIK BRACONIER,² ANDERS ISAKSSON¹
and LEIF JANSSON¹

From the Departments of ¹Clinical Chemistry and ²Infectious Diseases, University Hospital, Lund, Sweden

ABSTRACT. The lysosomal enzyme β -hexosaminidase was elevated in different forms of hepatitis. The enzyme level was correlated to the aminotransferases in the acute stage of hepatitis A and B. In hepatitis B there was also a correlation to ALP and bilirubin. In chronic hepatitis B a significant correlation was found only to ALAT. The elevated β -hexosaminidase levels are attributed to defective non-parenchymal liver cell function in hepatitis.

INTRODUCTION

The role of liver sinusoidal cells in many disease processes is still unclear (12). New results obtained by electron microscopy, cytochemistry and biochemistry have provided evidence for 4 separate sinusoidal cells: endothelial cells, Kupffer cells, fat-storing cells and pit cells (12).

Indirect data have been collected by studying reticuloendothelial system (RES) clearance rate of e.g. immunologically inert particles. The RES has in consequence been incriminated as the main clearance organ, taking care of foreign particles as well as immune complexes and endotoxins in the blood (8, 12). Endotoxemia has been described in viral hepatitis (8) and is known to occur in chronic active hepatitis (8).

The interaction of sinusoidal cells with hepatitis virus and the reaction of these cells during parenchymal cell necrosis remain to be investigated. The importance of the hepatic RES in the response to some hepatotropic viruses is assumed and the capacity of viruses to replicate within Kupffer cells is considered to influence some forms of hepatitis (2, 5, 9, 12).

Earlier studies (10, 11) have shown that the clearance of β -hexosaminidase, a lysosomal enzyme, from blood is performed by non-parenchymal cells of the liver. Specific surface cell receptors recognize mannose/glucosamine molecules on the carbohydrate chains of certain glycoproteins such as β -hexosaminidase. In an earlier study (7) we have shown that the β -hexosaminidase level is increased

in alcoholic intoxication, presumably because of RES depression.

In this communication we have investigated the 'endogenous clearance' of β -hexosaminidase in different forms of hepatitis.

MATERIAL AND METHODS

Patients

Hepatitis A. Serum samples were obtained from 15 patients aged 25-70 years admitted to the clinic for infectious diseases, Central Hospital, Växjö because of hepatitis A. 10 of the patients belonged to a common source outbreak. All patients had the typical clinical features of hepatitis A and also serological evidence of the disease (5). Samples were taken 1-2 weeks (Fig. 1 A), 2-4 weeks (Fig. 1 B), and 4-6 weeks (Fig. 1 C) following the onset of the disease.

Hepatitis B. Serum samples were obtained from 15 patients with hepatitis B aged 15-37 years and admitted to the clinic for infectious diseases, University Hospital, Lund. All gave a history fully consistent with hepatitis B and all had serological evidence of the disease (2). Samples from patients placed in the group acute hepatitis B had been taken within 8 weeks of onset and they should also show HBsAg-antigen activity and/or increased aminotransferase activity. Samples were taken after 1-2 weeks (Fig. 2 A), 2-4 weeks (Fig. 2 B), and 4-8 weeks (Fig. 2 C) after inception of the disease.

Patients who had lost serum HBsAg in samples taken more than 8 weeks after onset were confined to the group presented in Fig. 2 D and Table III.

Patients with consistent HBsAg over a long period (>1 year) were defined as chronic hepatitis B cases (Table IV and Fig. 2 E). All had the typical liver histology of chronic hepatitis.

HBsAg-negative patients with a biopsy verified chronic active hepatitis are presented in Fig. 2 F and Table V.



Fig. 1. Mean serum level and standard deviation of β -hexosaminidase (O), bilirubin (□), ALAT (Δ), and ALP ($\times$) in hepatitis A. Samples taken within 1-2 weeks (A), 2-4 weeks (B), and 4-6 weeks (C) after the onset of disease. D= controls (only the upper reference limit is given for the routine parameters).

Methods

Bilirubin (reference range 3-20 μ mol/l), aspartate aminotransferase (ASAT) (<0.7 μ kat/l), alanine aminotransferase (ALAT) (<0.7 μ kat/l), alkaline phosphatase (ALP) (<4.7 μ kat/l), and γ -glutamyltransferase (GT) (<1.0 μ kat/l), were determined as described earlier (7).

The β -hexosaminidase (E.C.3.2.1.30) assay was performed as follows: 50 μ l *p*-nitrophenyl-2-acetamidodeoxy- β -D-glucopyranoside (Koch-Light Labs. Ltd., Colnbrook, England), 7.5 mM in water, and 50 μ l 1 M citrate buffer, pH 4.0, and 100 μ l serum were incubated at 37°C for 30 min. The reaction was stopped with 3 ml of 0.2 M glycine buffer, pH 10.7, and read within 30 min in a spectrophotometer at 400 nm. *p*-nitrophenol was used as the standard. The amount of enzyme is defined as μ mole substrate split per min (U) per litre serum.

For statistical evaluation of the results, means and standard deviations were calculated. The different analyses were compared with each other using linear regression analysis and the coefficient correlations were tested for significance with Student's *t* test. Only $p < 0.05$ is regarded as significant.

Table I. Serum findings in acute hepatitis A ($n=45$)

NS = not significant

	Mean $\pm$ S.D.	
β -Hexosaminidase	28.9 $\pm$ 10.1	
Bilirubin	131 $\pm$ 118	
ALP	5.8 $\pm$ 3.8	
GT	2.2 $\pm$ 1.8	
ASAT	11.9 $\pm$ 17.6	
ALAT	19.0 $\pm$ 22.1	
	Correlation coefficient	<i>p</i>
β -Hexosaminidase/Bilirubin	0.22	NS
ALP	-0.23	NS
GT	0.02	NS
ASAT	0.40	<0.02
ALAT	0.44	<0.01

Table II. Serum findings in acute hepatitis B ($n=45$)

NS = not significant

	Mean $\pm$ S.D.	
β -Hexosaminidase	25.6 $\pm$ 12.7	
Bilirubin	64.1 $\pm$ 91.3	
ALP	6.3 $\pm$ 2.5	
GT	4.8 $\pm$ 8.7	
ASAT	12.6 $\pm$ 12.6	
ALAT	19.0 $\pm$ 18.8	
	Correlation coefficient	<i>p</i>
β -Hexosaminidase/Bilirubin	0.61	<0.001
ALP	0.40	<0.02
GT	0.14	NS
ASAT	0.46	<0.005
ALAT	0.33	<0.05

RESULTS

Hepatitis A

Fig. 1 indicates that β -hexosaminidase is increased in hepatitis A and that it decreases slowly compared to the aminotransferases. Examination of patients with hepatitis A more than 3 months after the onset showed normal levels of β -hexosaminidase and the other routine parameters.

Table I shows that there was a correlation between β -hexosaminidase and the aminotransferases but not to the other routine tests. Bilirubin was not correlated to the aminotransferases or ALP, but ALP and GT showed good correlation ($p < 0.001$).

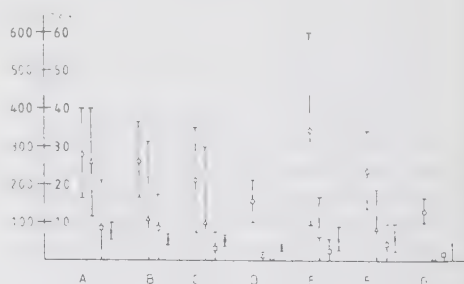


Fig. 2. Mean serum level and standard deviation of β -hexosaminidase (O), bilirubin (□), ALAT (Δ), and ALP ($\times$) in different forms of hepatitis B and chronic active hepatitis. Samples taken within 1-2 weeks (A), 2-4 weeks (B), and 4-8 weeks (C) after onset of hepatitis B. D= controls after acute hepatitis B (more than 8 weeks after onset). E= chronic hepatitis B, F= chronic active hepatitis. G= controls (only the upper reference limit is given for the routine parameters).

Table III. Serum findings in patients after hepatitis B (HBsAg-negative and more than 8 weeks after onset) (n=18)

NS = not significant

	Mean $\pm$ S.D.		
β -Hexosaminidase	16.5 $\pm$ 5.6		
Bilirubin	10.9 $\pm$ 3.4		
ALP	3.5 $\pm$ 0.8		
GT	0.8 $\pm$ 0.5		
ASAT	0.7 $\pm$ 0.5		
ALAT	1.0 $\pm$ 1.1		
		Correlation coefficient	p
β -Hexosaminidase/Bilirubin		-0.02	NS
ALP		-0.20	NS
GT		0.30	NS
ASAT		-0.04	NS
ALAT		-0.10	NS

Hepatitis B

β -Hexosaminidase shows the same course in hepatitis B as in hepatitis A during the acute period (i.e. HBsAg-positive and/or increased level of the aminotransferases within 8 weeks after onset), while the aminotransferases do not decrease as rapidly as in hepatitis A (Fig. 2). Table II shows a good correlation between β -hexosaminidase and ASAT, ALAT, bilirubin, and ALP. In acute hepatitis B there was a good correlation between the aminotransferases and bilirubin ($p < 0.05$). There was no significant correlation between ALP and GT or ALP and bilirubin.

The controls after acute hepatitis B (>8 weeks after onset) showed slightly increased aminotransferases and raised levels of β -hexosaminidase. Table III indicates that there was no correlation between β -hexosaminidase and the other tests.

Chronic active HBsAg-positive hepatitis B

Fig. 2 demonstrates a great variation of β -hexosaminidase and abnormal elevation of the other routine liver tests. Table IV shows a good correlation between β -hexosaminidase and ALAT.

Chronic active hepatitis

An increased level of β -hexosaminidase was found and the other liver tests were also raised (Fig. 2). In this small number of individuals one could not find any significant correlation between β -hexosaminidase and the other routine parameters (Table V).

Table IV. Serum findings in chronic hepatitis B (HBsAg-positive) (n=12)

NS = not significant

	Mean $\pm$ S.D.		
β -Hexosaminidase	34.4 $\pm$ 26.6		
Bilirubin	26.1 $\pm$ 32.2		
ALP	5.8 $\pm$ 3.5		
GT	3.7 $\pm$ 4.5		
ASAT	2.5 $\pm$ 2.2		
ALAT	7.0 $\pm$ 10.3		
		Correlation coefficient	p
β -Hexosaminidase/Bilirubin		0.51	NS
ALP		0.21	NS
GT		0.46	NS
ASAT		0.49	NS
ALAT		0.74	<0.005

Other forms of hepatitis

Seven patients with hepatitis due to infectious mononucleosis, 4 patients with halothane hepatitis and 1 patient with hepatitis due to lues were also investigated. All these patients showed an increased level of β -hexosaminidase with the peak during the most acute stage of their disease. The level of β -hexosaminidase was of the same magnitude as in acute hepatitis A and B, although transaminases showed a less marked increase in these forms of hepatitis.

DISCUSSION

As mentioned in the introduction, the interference of sinusoidal cells by virus is an almost unexplored

Table V. Serum findings in chronic active hepatitis (HBsAg-negative) (n=17)

NS = not significant

	Mean $\pm$ S.D.		
β -Hexosaminidase	24.0 $\pm$ 9.5		
Bilirubin	45.5 $\pm$ 52.3		
ALP	6.3 $\pm$ 3.7		
GT	3.2 $\pm$ 2.6		
ASAT	10.3 $\pm$ 18.4		
ALAT	8.6 $\pm$ 9.9		
		Correlation coefficient	p
β -Hexosaminidase/Bilirubin		-0.01	NS
ALP		-0.08	NS
GT		0.33	NS
ASAT		0.05	NS
ALAT		-0.01	NS

field of investigation. Very low doses of frog virus FV3 produce severe toxic reaction of sinusoidal cells shortly after injection (6). There is however evidence that both hepatitis A and B viruses are without cytopathic effect and that the liver cell damage is the result of an immune-mediated mechanism (2, 5). This concept makes a direct effect of hepatitis virus on Kupffer cells unlikely. The theory of immunological damage could also be applied to the cases of chronic active hepatitis.

Factor VIII antigen (VIII_{AGN}) is probably cleared by Kupffer cells and is increased in early stages of viral hepatitis in man, which is indirect evidence for an early decreased phagocytic capacity (3). Phagocytic activity is increased in acute liver disease such as hepatitis 3 weeks after onset of icterus (4).

The increased frequency of endotoxemia in hepatitis (8) also supports the theory that sinusoidal cells are affected in this disease and that there is a dysfunction of the clearance system. The accumulating evidence for the pathogenetic significance of endotoxins in various liver diseases (8) has therapeutic implications. One approach may be RES stimulation to increase clearance of endotoxins. The injection of aluminum hydroxide particles has been reported to influence favourably the course of hepatitis A and B (1). After injection into rats, the particles are exclusively found in Kupffer cells (12).

An increase in β -hexosaminidase in hepatitis has been noted in one earlier study (13). In our investigation a slow clearance of β -hexosaminidase was noted. In hepatitis A and B there was a good correlation between β -hexosaminidase and the aminotransferases. Elevated aminotransferases are considered typical of the active stage of the hepatitis. In hepatitis B there was also a correlation between β -hexosaminidase and bilirubin. This correlation was not seen in hepatitis A. One possible explanation of this finding could be that no correlation existed between the aminotransferases and bilirubin in hepatitis A, where the aminotransferases decreased rapidly compared to bilirubin. The decrease of aminotransferases and bilirubin was more parallel in hepatitis B.

A correlation between β -hexosaminidase and the aminotransferases was also found in alcoholic liver disease (7). However, there is no proportionality between levels of aminotransferases and β -hexosaminidase. In alcoholic liver disease the mean

levels of ASAT and ALAT were 1.79 and 1.35 μ kat/l, respectively, while the mean level of β -hexosaminidase was 35.9 U/l. In the present study the mean aminotransferase levels were much higher while the mean β -hexosaminidase level was lower than in alcoholic liver disease. These findings give support to the theory that an increased level of β -hexosaminidase in liver disease is not due to cell leakage of the enzyme but is related to defective clearance.

The defective clearance of β -hexosaminidase could thus be attributed to a direct toxic effect of the virus on the non-parenchymal cells of the liver or the blocking of the RES by virtue of overload by large amounts of circulating immune complexes (2) or necrotic parenchymal cells.

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Address for reprints:

B. Hultberg, M.D.,
Inst. för klinisk kemi,
Lasarettet,
S-221 85 Lund,
Sweden

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Influence of Ethanol on the Human Serum Level of Beta-Hexosaminidase

*Anders Isaksson^a, Claes Blanche^b, Björn Hultberg^a, Bo Joelsson^c*Departments of ^aClinical Chemistry and ^cSurgery, University Hospital, Lund;^bPharmacia Health Care, Uppsala, Sweden

Key Words. Alcohol · Alcoholic liver cirrhosis · Chronic alcoholics · Lysosomal enzymes · Serum · Teetotalers

Abstract. Serum activities of the lysosomal enzymes β -hexosaminidase, α -fucosidase and α -mannosidase in teetotalers, healthy persons with 'ordinary' alcohol consumption, chronic alcoholics and patients with alcoholic liver cirrhosis were compared. There was no difference between controls and teetotalers, whereas serum from chronic alcoholics and patients with alcoholic liver cirrhosis showed a significant increase of lysosomal enzymes compared to controls and teetotalers.

The effect of daily alcohol ingestion on the serum activity of lysosomal enzymes was studied in 9 healthy volunteers. A minor but significant increase was found for β -hexosaminidase. It is concluded that the increase of lysosomal enzyme activity in serum from alcoholics is due to the liver dysfunction.

Introduction

Activities of several lysosomal hydrolases are found in human serum. Precursors of lysosomal enzymes are synthesized in several cell types and processed to mature tissue forms in the lysosomes [1]. In cultured cells a small fraction of the precursors escapes segregation into the lysosomes and is secreted into the culture medium [1]. A similar mecha-

nism appears to be responsible for human serum enzyme activity [2].

Tissue forms of lysosomal enzymes are rapidly cleared from both rat and human blood circulation when injected intravenously [3, 4]. The uptake in the rat is mediated by the mannose/N-acetylglucosamine receptors on the non-parenchymal liver cells [3]. In contrast, sialylated serum β -hexosaminidase (β -hex) forms exhibit a slow clear-

ance, which is, however, enhanced by desialylation [5]. Possibly, the serum forms like other glycoproteins following desialylation are cleared via the galactose receptor on the hepatocytes [5].

Increased serum activities of β -hex are found in patients with liver disease [6] and in chronic alcoholics with acute ethanol intoxication [7]. Augmented production or decreased elimination of the enzyme alone or in combination might cause this increase. It is not known if alcohol per se has an effect on the enzyme activity. In order to elucidate the effect of ethanol on the serum levels of lysosomal enzymes, enzyme levels in healthy volunteers ingesting alcohol were compared to those of teetotalers, healthy persons with 'ordinary' alcohol consumption, chronic alcoholics and patients with alcoholic liver cirrhosis.

Material and Methods

Study groups

Group A comprised 20 healthy persons (10 females, 10 males; median age 29, range 23–47 years) with ordinary alcohol consumption. Their median alcohol consumption during the week before fasting blood samples were obtained was 42 g alcohol per week (range 0–312 g) as stated by themselves.

Group B consisted of 17 teetotalers (4 females, 13 males; median age 40, range 18–71 years).

Group C comprised 17 chronic alcoholics as defined by *Jellinek* [8] (4 females, 13 males; median age 47, range 27–69 years). According to their own estimation, their median alcohol consumption during the week before fasting blood samples were obtained was 700 g alcohol per week (range 0–2,870 g). No liver biopsy was performed in these patients. According to previous studies [9], most of them probably had at least liver steatosis.

Group D comprised 28 patients with biopsy-proven alcoholic liver cirrhosis (8 females, 20 males; median age 48, range 28–71 years). Following at least

4 days with regular hospital food and total alcohol abstinence, overnight fasting blood samples were obtained.

Group E consisted of 9 apparently healthy volunteers (4 females, 5 males; median age 32, range 23–36 years) who ingested 60 g alcohol daily during a period of 10 days. Fasting blood samples were obtained at days 0, 3, 6, 10, 14, 18 and 25. Their median alcohol consumption in the week before the start of the blood sampling period was 196 g (range 94–342 g). During 10 days before the start of the experimental period they completely avoided consumption of alcohol.

Assay Procedures

Serum activities of β -hexosaminidase (EC 3.2.1.30), α -fucosidase (EC 3.2.1.51) at pH 5.5 and α -mannosidase (EC 3.2.1.24) at pH 4.5 were determined as described earlier [10, 11]. One unit of enzyme activity represents 1 μ mol of substrate split per minute. Enzymatic determination of total serum bile acids was performed using the stereognost-3 α RRA (Nyegaard & Co., Oslo, Norway). Bilirubin, alkaline phosphatase (ALP), γ -glutamyl transferase (GT), aspartate-aminotransferase (ASAT) and alanine-aminotransferase (ALAT) were determined using standard methods, and the different proteins (albumin, prealbumin, orosomucoid, ceruloplasmin, α_1 -antitrypsin, haptoglobin, IgG, IgA, IgM) were determined by immunoelectrophoresis as described earlier [12].

Statistical Analysis

The Mann-Whitney test for unpaired data and Wilcoxon's rank sum test for paired data were used for statistical analysis.

Results

β -Hexosaminidase, α -mannosidase and α -fucosidase activities were significantly increased in serum from patients with alcoholic cirrhosis (group D) compared to controls (group A) and teetotalers (group B) (table I). Likewise, β -hex and α -mannosidase were significantly increased in serum from chronic alcoholics (group C). The median value of

Table I. Serum enzyme activities¹

	n	β -Hexosaminidase	α -Mannosidase	α -Fucosidase	ASAT	GT
Controls with 'ordinary' alcohol consumption	20	13.1 (10.4–20.8)	0.71 (0.56–1.02)	4.6 (2.5–7.4)	0.3 (0.1–0.4)	0.2 (0.1–0.8)
Teetotalers	17	14.5 (NS) (11.5–21.9)	0.79 (NS) (0.56–1.13)	4.4 (NS) (2.3–7.9)	0.2 (NS) (0.1–0.5)	0.2 (NS) (0.1–0.7)
Chronic alcoholics	17	32.3* (12.7–53.7)	1.21* (0.68–1.87)	6.0 (NS) (2.9–9.8)	1.4* (0.3–4.9)	1.7* (0.2–11.6)
Alcoholic liver cirrhosis	28	27.3* (13.1–43.1)	1.36* (0.51–2.10)	8.2* (2.1–12.2)	0.9* (0.3–3.5)	2.9* (0.6–11.9)

¹ Median and range are given. Activities are expressed as U (μ mol substrate split per min) per liter serum for the lysosomal enzymes and μ kat per liter serum for ASAT and GT. Statistical significance was assessed by the Mann-Whitney test for unpaired data.

* $p < 0.05$; NS = Not significant.

α -fucosidase was elevated in serum from chronic alcoholics but did not reach statistical significance.

Many tests have been proposed for the detection of heavy drinking or alcoholic liver injury at an early stage. As GT and ASAT probably are the most widely used, they were also analysed in groups A–D for comparison with the lysosomal enzymes (table I). Both GT and ASAT were significantly increased in groups C and D compared to groups A and B.

In the group of volunteers (E) ingesting alcohol, a minor but significant rise was seen in serum β -hex activity (about 25% compared to the initial value) at the first sample occasion (table II) (i.e., 3 days after the start of the experiment). Four days after withdrawal of ethanol the β -hex activity was normalized (table II). α -Fucosidase and α -mannosidase showed similar increments during alcohol ingestion but the differences did not reach statistical significance. Lysosomal se-

rum enzyme activities before the start of the experimental period (day –10) were in the same range as during the period of standardized alcohol ingestion. In this group (E) no significant changes in GT or ASAT were seen during the experiment (table II), nor did the other routine serum liver tests, bile acids and serum proteins show any significant alterations during the experimental or follow-up period.

Discussion

The present study shows that alcohol ingestion in the presence of normal liver function causes a small but significant increase in serum β -hex. This increase, however, is far from that exhibited by patients with alcoholic liver cirrhosis or chronic alcoholics with presumed liver steatosis. These findings indicate that liver dysfunction is the main factor causing the enzyme changes. This con-

Table II. Serum levels of β -hex, ASAT and GT in 9 healthy volunteers during 10 days of alcohol ingestion and in the following 15 days of abstinence¹

Day	β -hex	ASAT	GT
-10	15.4* (10.4-23.1)	0.2 (0.1-0.5)	0.2 (0.1-0.4)
0	12.9 (7.6-16.1)	0.3 (0.1-0.6)	0.2 (0.1-0.4)
3	15.2* (10.1-19.1)	0.2 (0.1-0.4)	0.2 (0.1-0.3)
6	16.1* (9.7-19.4)	0.2 (0.1-0.5)	0.2 (0.1-0.3)
10	16.1* (8.8-20.1)	0.2 (0.1-0.4)	0.2 (0.1-0.4)
14	12.9 (8.1-15.7)	0.2 (0.1-0.4)	0.2 (0.1-0.4)
18	12.5 (8.5-16.1)	0.2 (0.1-0.5)	0.2 (0.1-0.3)
25	12.7 (7.6-16.6)	0.2 (0.1-0.5)	0.2 (0.1-0.4)

¹ Median and range are given. Activities are expressed as U per liter serum for β -hex and μ kat per liter serum for ASAT and GT. Statistical significance was assessed by Wilcoxon's rank sum test for paired data.

* $p < 0.05$, significance compared to day 0.

cept is supported by our previous study showing that patients with cirrhosis of alcoholic and non-alcoholic origin have equally increased serum levels of β -hex [12]. Furthermore, following alcohol withdrawal, volunteers with normal liver function exhibit a return to normal serum β -hex activities within 4 days. In contrast, previously studied chronic alcoholics [7] with presumed liver steatosis on the 7th day of abstinence still showed increased activities.

Since the ingestion of 60 g alcohol daily only exerted a relatively small effect on the serum level of lysosomal enzymes it is not surprising that there was no significant difference between total abstainers and persons with 'ordinary' alcohol consumption.

The significant increase of serum lysosomal enzyme activities found in both chronic alcoholics and in patients with alcoholic liver cirrhosis confirms earlier reports of increased serum levels of lysosomal enzymes in alcoholic liver disease [7, 13, 14]. In contrast to serum, the specific activities of most lysosomal enzymes in the human liver are unaffected by alcoholic liver disease [15, 16].

Lysosomal enzymes and routine liver tests (ASAT, GT) were increased to a similar extent in chronic alcoholics compared to patients with liver cirrhosis. In the group of normal volunteers ingesting alcohol, several tests (ASAT, ALAT, GT, ALP, bile acids, and serum proteins) considered to be influenced by liver dysfunction were analysed. In contrast to serum β -hex, none of these showed any significant changes during the experiment. Thus, it would seem that serum β -hex more sensitively reflects alcohol ingestion than any of the other tests.

Recently, we have shown that the increase of serum β -hex activity in chronic alcoholics is mainly caused by the serum isoenzyme Hex-P [17]. Elimination of Hex-P in the rat's circulation does not differ from that of other serum isoenzymes of β -hex [5; own unpublished findings]. It seems likely that the increase of serum Hex-P is caused by increased enzyme production, possibly from macrophages, as suggested earlier [12, 17].

In conclusion, the increased serum β -hex in chronic alcoholics probably depends upon the alcohol-induced liver dysfunction and

not on a direct effect of the drug. However, serial determinations of β -hex may be useful to monitor patients with alcohol-related diseases. Alcohol ingestion would cause minor increases, while major changes in the serum activity should give rise to a suspicion of liver damage.

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Björn Hultberg, MD,
Department of Clinical Chemistry,
University of Lund,
S-221 85 Lund (Sweden)

Isoenzyme Pattern of Serum β -Hexosaminidase in Liver Disease, Alcohol Intoxication, and Pregnancy

B. Hultberg, A. Isaksson

Department of Clinical Chemistry, University Hospital, Lund, Sweden

Key Words. Ethanol intoxication · Isoenzymes · Liver disease · Pregnancy · Serum β -hexosaminidase

Abstract. The isoenzyme distribution of serum β -hexosaminidase was studied in 11 pregnant women, 18 patients with cirrhosis of the liver, 14 patients with cholestasis, and 9 chronic alcoholics with acute ethanol intoxication. Serum β -hexosaminidase activity was elevated in sera from all groups. The results of isoenzyme separation by isoelectric focusing demonstrated that the isoenzyme pattern in the different patient groups was similar to pregnancy with a preferential increase of isoenzyme forms with pI's between 5.6 and 6.8.

Introduction

The human lysosomal hexosaminidases (N-acetyl- β -D-glucosaminidases, abbreviated Hex, EC 3.2.1.30) are a complex group of glycoprotein isoenzymes. Listed in order of decreasing isoelectric points, they are named Hex B, I₁, I₂, P, A, and S [1–4]. Hex I (I₁ and I₂) are intermediate heat-stable forms normally present in serum which show a considerable increase in patients with Tay-Sachs disease [3]. A chromatographically indistinguishable component with similar thermal stability properties increases dramatically during pregnancy and has been termed Hex P [2–8]. There is at present no method available to distinguish between the intermediate forms and Hex P.

It has been previously shown that total serum Hex activity is increased in pregnancy

[6–8], acute ethanol intoxication [9], cholestasis [10], and several other liver diseases [10–12]. In all conditions preliminary results have indicated that this increase was mainly due to an increase of isoenzyme P [6]. In this communication we have extended the studies concerning the isoenzyme pattern in serum from patients with increased Hex activity.

Material and Methods

Study Population. The diagnosis of patients with hepatobiliary diseases was based on the presence of typical clinical, serum biochemical (table I), hepatic-histological and, where appropriate, cholangiographical findings. All patients' sera were found to be negative for hepatitis-B surface antigen. Of the cirrhosis patients 10 had alcoholic cirrhosis and 4 cirrhosis caused by hepatitis. In 4 patients the cause of cirrhosis was unknown (cryptogenic). 8 patients with cholestasis had malignant processes in the biliary duct or pan-



Table 1. Serum Hex activity, bilirubin and 'liver enzymes' (mean $\pm$ SD) in patients with acute ethanol intoxication, liver diseases, and in pregnancy

	Hex	Bil	ALP	GT	ASAT	ALAT
Acute ethanol intoxication	34.1 $\pm$ 11.1	16.1 $\pm$ 14.4	—	1.98 $\pm$ 1.45	1.12 $\pm$ 0.90	1.27 $\pm$ 0.98
Cholestasis	50.8 $\pm$ 11.7	246 $\pm$ 158	26.0 $\pm$ 11.4	10.5 $\pm$ 8.8	1.78 $\pm$ 0.50	0.95 $\pm$ 0.45
Cirrhosis	31.0 $\pm$ 11.6	22.9 $\pm$ 8.4	14.9 $\pm$ 11.4	5.0 $\pm$ 4.3	1.40 $\pm$ 0.91	0.83 $\pm$ 0.72
Pregnancy	61.1 $\pm$ 32.0	—	—	—	—	—
Controls	12.5 $\pm$ 3.6	3–20	0.8–4.6	< 1.0	< 0.7	< 0.7

Hex activity is expressed in μ mol substrate hydrolyzed/min/l. The concentration of Bil (bilirubin) is in μ mol/l and the activities of ALP (alkaline phosphatase), GT (γ -glutamyl transferase), ASAT (aspartate aminotransferase), and ALAT (alanine aminotransferase) are expressed in μ kat/l.

creas, and 6 patients had severe forms of choledocholithiasis. All patients had been icteric for more than 1 week before sampling. The 9 patients with acute ethanol intoxication were all chronic alcoholics and have been described before [9].

The 11 pregnancies (5 women from second and 6 from third trimester) were uncomplicated.

Enzyme Assay. The determination of Hex activity was performed as described before [10]. Fractions from the isoelectric column were analyzed as outlined in the legend to figure 1.

Isoelectric Focusing. This was performed on a 110-ml column, using ampholine with a pH range of 4–6 (LKB Products, Stockholm, Sweden). The time for electrofocusing was 42 h. At the end of the experiment the voltage and currents were 550 V and 0.8 mA, respectively. 2-ml fractions were collected.

Statistics. The means and standard deviations were calculated. The different parameters were compared with linear regression analysis and the correlation coefficients were tested for significance with Student's *t* test. Only $p < 0.05$ was regarded as significant.

Results

The isoenzymes in the different pI ranges (fig. 1) were dialyzed over night and subjected to ion-exchange chromatography on DEAE-cellulose as described by several

workers [3–5, 7]. The activities at pI 4.2–4.8, pI 4.9–6.8, and pI $\geq$ 6.9 were mainly (> 80% of each activity) chromatographed as Hex A, Hex I, and Hex B [3–5, 7], respectively.

Hex activity was significantly increased in pregnant women and the different patient groups (table I). Although all the isoenzyme forms were increased, the isoenzymes in the pI range 5.6–6.8 were preferentially increased (fig. 1).

The correlations between total serum Hex activity and the relative distribution of the activities within different pI ranges are shown in table II as well as figures 2 and 3. Total serum enzyme activity showed a positive correlation to enzyme activity in the pI range 5.6–6.8 (mainly intermediate or P forms) and to the activity above pI 5.6 (intermediate, P, and B forms). A negative correlation was seen between total serum enzyme activity and activity between pI 4.2 and 4.8 (mainly A forms). There was no significant correlation in the total serum enzyme activity to the activity above pI 6.9 (mainly β -hexosaminidase B). Similar findings were observed in the different subgroups of patients and pregnant women.

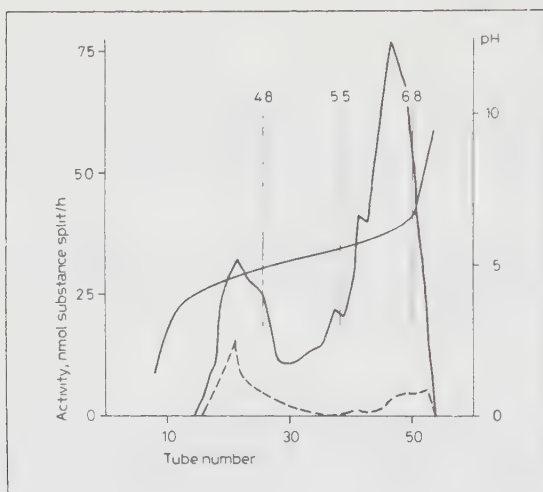


Fig. 1. Isoelectric focusing of Hex in serum from 1 control (---) and a pregnant woman in the third trimester (—). The conditions used for isoelectric focusing were as described in 'Materials and Methods'. For enzyme assay, 100 μ l of column fractions were incubated with 100 μ l of 1 mmol/l solution of 4-methylumbelliferyl- β -D-2-acetamido-2-deoxyglucopyranoside dissolved in citrate-phosphate buffer, pH 4.5 (100 mmol/l citric acid, 200 mmol/l Na_2HPO_4).

Table II. Correlations between total serum Hex activity and activity of different isoenzyme forms

	Correlation coefficient	Significance
Total Hex/activity		
pI 4.2–4.8	–0.66	<0.001
pI 4.9–5.5	–0.08	NS
pI 5.6–6.8	0.57	<0.001
pI > 5.6	0.65	<0.001
pI > 6.9	0.17	NS

Discussion

Increased activity of several lysosomal hydrolases in serum from pregnant women [6] and liver disease [unpublished results] have been observed. The present study confirms that Hex activity is elevated in serum from pregnant women and patients with acute ethanol intoxication as well as different forms of liver disease. Correlation studies between the

isoenzyme forms and total Hex revealed that increase was mainly due to the isoenzyme forms with pI's between 5.6 and 6.8. The cause for the increased serum Hex activity is not known, but it is possible that either efflux or elimination of the enzyme are altered.

The elimination of some glycoproteins from circulation has been ascribed to carbohydrates as recognition markers. For instance, infused lysosomal hydrolases (tissue forms) and glycoproteins with mannose or N-acetylglucosamine as the terminal residues are removed rapidly (half-life of 5–10 min) from the circulation by the liver where uptake is mediated by receptors on the nonparenchymal liver cells [13–15]. Although the tissue and serum forms of Hex are related, they differ in physicochemical properties [4]. It is probable that their eliminations differ, as suggested by *Bearpark and Stirling* [16], who showed that infused serum Hex A was eliminated rapidly from circulation only after de-

Fig. 2. The relative distribution of Hex activity between pI 4.2 and 4.8 compared to total serum activity in different conditions. 9 controls exhibited values which fell within the shaded area (■). ■ = Pregnant women; ▲ = patients with acute ethanol intoxication; ● = patients with cirrhosis; ○ = patients with cholestasis.

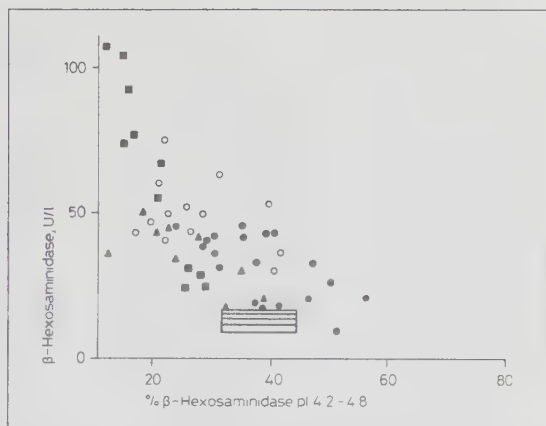
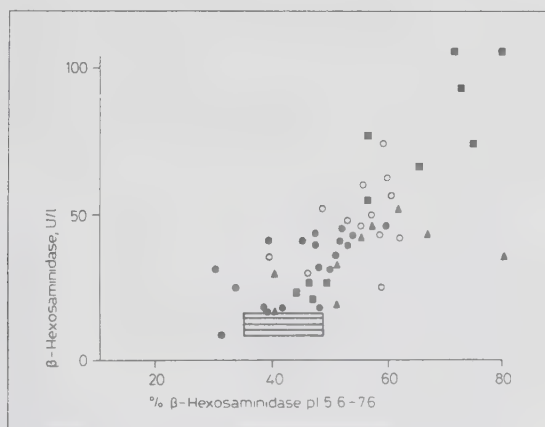


Fig. 3. The relative distribution of Hex activity between pI 5.6 and 7.6 compared to total serum activity in different conditions. Legends as in figure 2.



sialylation. On the other hand, the native sialylated forms were not eliminated under the experimental conditions used.

Increased production of lysosomal hydrolases followed by efflux could also account for the increase of serum Hex activity. The macrophages could serve as the cellular source, since they have been shown to be cells which have responded to different stimuli by

increasing both the intracellular contents and release of lysosomal hydrolases [17]. Furthermore, *Schrecker et al.* [18] found that the serum activities of lysosomal hydrolases could serve as biochemical markers of macrophage activation in mice. Likewise, *Tanner et al.* [19] found that both the intracellular activity and release of Hex were increased in macrophages isolated from the liver of rats

treated with *Corynebacterium parvum*. The total body macrophages in liver disease might be activated by enteric antigens bypassing the filtering action of the liver. This concept is supported by the findings of Ganguly et al. [20], who showed that blood monocytes from patients with chronic liver disease exhibited increased intracellular content and release of Hex. Sources other than macrophages have also been proposed. For instance, a good correlation for Hex activity and the histological grade of liver fibrosis was shown in biopsies from cirrhotic liver [21].

A parallel to the proposed phenomenon of antigenic substances bypassing the filtering action of the liver in chronic liver disease exists in pregnancy, where substances of fetal origin may enter maternal circulation. It is equally feasible that elevated concentrations of estrogens are responsible for macrophage activation [22].

The fact that the isoenzyme pattern in pregnancy, acute alcohol intoxication, and liver disease is similar indicates a common mechanism responsible for the increase in Hex. This mechanism might represent an activation of the macrophages resulting in increased synthesis and release of the enzyme.

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Björn Hultberg,
Department of Clinical Chemistry,
University Hospital,
S-221 85 Lund (Sweden)

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Activity of Lysosomal Hydrolases in Plasma at Term and Post Partum

A. Isaksson^a, B. Gustavii^b, B. Hultberg^a, P. Masson^a

^aDepartment of Clinical Chemistry and

^bDepartment of Obstetrics and Gynecology, University Hospital, Lund, Sweden

Key Words. Hydrolases · Isoenzymes · Plasma · Postpartum period · Third pregnancy trimester

Abstract. The activity and isoenzyme pattern in plasma of β -hexosaminidase (abbreviated Hex) and four other lysosomal hydrolases (α -fucosidase, β -glucuronidase, α -hexosaminidase, α -mannosidase) were studied in 50 women at term and in 10 women at various intervals during the first 6 days after parturition. All hydrolases had elevated activity at term, compared with controls. After parturition the activity of α -mannosidase returned to the normal level within 2 days and that of Hex, β -glucuronidase, and α -hexosaminidase within 6 days; α -fucosidase having a slightly elevated activity even at the end of this period. Isoenzyme analysis by isoelectric focusing was informative only in the case of Hex. Thus, the increased activity of Hex at term was mainly due to an increase in the isoenzyme form(s), with pI(s) between 5.6 and 6.8. The enzyme pattern of Hex during pregnancy and post partum observed in this study seems to have certain similarities to the previously noted enzyme pattern of Hex in acute ethanol intoxication and following withdrawal of ethanol. As lysosomal membranes are labilized by elevated levels of steroids, it is of interest to note that high levels of these hormones are found in plasma both at term and in chronic liver disease.

Introduction

The activity of β -hexosaminidase (2-acet-amido-2-deoxy- β -D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30, abbreviated Hex) increases in the plasma of pregnant women towards term [1, 2]. No correspond-

ing increase in amniotic fluid and placental tissue has been observed [2–4]. The activity has been found to return to the normal level within a week after parturition [1, 3].

We have previously described how an increase in plasma Hex was also found in various liver disorders, such as liver cirrhosis,

Table I. Lysosomal hydrolase activity in plasma of women at term

Enzyme	Activity ¹		
	at term (n = 50)	controls (n = 30)	p value
α -Fucosidase	8.0 (1.0–14.9)	3.5 (0.5–5.9)	< 0.01
β -Glucuronidase	0.46 (0.08–0.87)	0.20 (0.06–0.48)	< 0.01
α -Hexosaminidase	7.1 (3.0–18.8)	4.5 (2.5–7.7)	< 0.01
β -Hexosaminidase	75 (34–140)	15 (7–21)	< 0.01
α -Mannosidase	2.0 (0.7–3.1)	0.8 (0.2–1.3)	< 0.01

¹ Median and range in micromoles of substrate split/min/l plasma.

cholestasis and acute ethanol intoxication [5, 6]. The increase in Hex in these conditions would seem to be due to an increase in the isoenzyme form designated Hex P [7]. The mechanism underlying the increase in plasma Hex activity is not known. In order to elucidate further the enzyme pattern of Hex, we have studied the activity and, in particular, the isoenzyme pattern of this enzyme in plasma of women at term and during the first 6 days after parturition. Four other hydrolases were studied in the same way.

Materials and Methods

EDTA plasma was obtained from 50 healthy women at term and from 10 healthy women at various intervals after parturition. EDTA plasma from 30 apparently healthy, nonpregnant women (blood donors and laboratory personnel) served as controls. All the samples were stored at -80°C until analyzed.

Enzyme analyses for Hex, α -mannosidase (α -D-mannoside mannohydrolase, EC 3.2.1.24) at pH 4.5

and α -hexosaminidase (2-acetamido-2-deoxy- α -D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.50) were performed as described earlier [8]. For α -fucosidase (α -L-fucoside fucosylhydrolase, EC 3.2.1.51), 25 μl of plasma were incubated at 37°C with 100 μl of a 1 mmol/l solution of 4-methylumbelliferyl- α -L-fucopyranoside (Koch-Light Laboratories Ltd, Colnbrook, Bucks, UK) in water, and 25 μl of a 1 mol/l sodium citrate buffer, pH 4.0. The incubation times were 30 and 60 min. The reaction was stopped by adding 3 ml of a 0.2 mol/l glycine buffer, pH 10.7 (0.2 mol/l glycine adjusted to pH with 1 mol/l NaOH). The fluorescence of 4-methylumbelliferone was read as described elsewhere [8]. For β -glucuronidase (β -D-glucuronide glucuronohydrolase, EC 3.2.1.31), 25 μl of the sample were incubated with 100 μl of a 1 mmol/l solution of 4-methylumbelliferyl- β -D-glucuronide (Koch-Light) in citrate-phosphate buffer (100 mmol/l citric acid, 200 mmol/l NaH_2PO_4), pH 4.5.

The incubation temperature, times and the rest of the procedure were as outlined for α -fucosidase.

For isoelectric focusing, 1 ml of plasma was applied to a 110-ml LKB column (LKB Produkter, Stockholm, Sweden), using ampholines with a pH range of 4–6. The samples were electrofocused for 42 h. At the end of the experiment, the voltage was 550 V, and current 0.8 mA. Fractions of 2 ml were

Table II. Lysosomal hydrolase activity in plasma of 10 women at various intervals after parturition

Enzyme	Activity ¹ at intervals after parturition					
	0.5 h	10–15 h	1 day	2 days	4 days	6 days
α -Fucosidase	9.6	8.4	7.6	6.7	5.0	4.5
β -Glucuronidase	0.38	0.34	0.31	0.28	0.23	0.19
α -Hexosaminidase	8.1	6.9	6.6	6.3	5.2	4.7
β -Hexosaminidase	85	52	39	27	18	14
α -Mannosidase	0.9	0.7	0.7	0.6	0.6	0.6

¹ Median in micromoles of substrate split/min/l plasma.

collected and, of these, 100 μ l were incubated at 37 °C with 100 μ l of a 1 mmol/l solution of 4-methylumbelliferyl glycoside dissolved in appropriate citrate-phosphate buffer. Hex was incubated for 2 h; the other enzymes for 20 h.

Wilcoxon's rank test was used for the statistical evaluation.

Results

The activity of all hydrolases studied was found to be increased at term (table I), most notably in the case of Hex.

The time taken for the increased level of enzyme activity to return to normal after parturition was less than 6 days, although α -fucosidase had a slightly elevated level even at the end of this period (table II). The increase in Hex activity at term was due mainly to the increase in the isoenzyme forms with pI values between 5.6 and 6.8. It was predominantly this form which decreased during the period following parturition (fig. 1). The

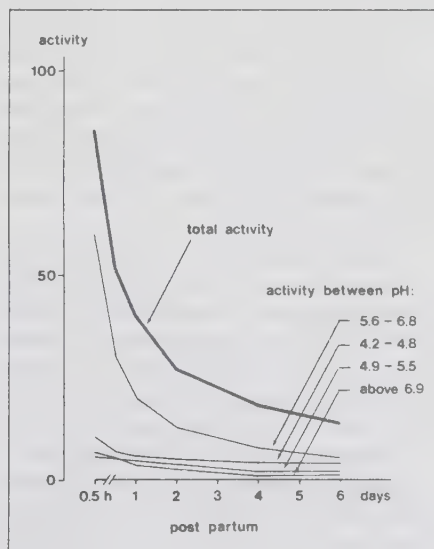


Fig. 1. Decrease after parturition in the activity of plasma Hex and its isoenzyme forms in 10 women. Median in micromoles of substrate split/min/l plasma.

other hydrolases showed no obvious change in their isoenzyme pattern during the postpartum period.

Discussion

Our observation of an increased activity of the lysosomal hydrolases studied, with Hex being the most increased, is in agreement with the findings made by others [1, 2].

Our isoelectric focusing studies showed that the large increase in total Hex activity in plasma of women at term was due to an increase of the form(s) of Hex with pI values between 5.6 and 6.8. This form was earlier designated as Hex P [9]. Its activity was found to decrease after parturition. Hex P activity is also increased in liver damage and alcoholic liver disease [7]. In the latter case, the overall Hex activity returned to the normal level after about a week following ethanol withdrawal [5]. Whether this decrease is also mainly due to Hex P requires further investigation.

Several explanations for the increase of lysosomal enzymes in pregnancy and chronic liver disease have been proposed. We feel that two of these explanations are feasible. First, an increased production and secretion of the enzymes due to macrophage activation [7], where Hex P is either a secretory form of the hexosaminidase and/or a precursor to mature hexosaminidase forms, as suggested by Hasilik [10]. Second, the lysosomal membrane may be labilized by elevated levels of steroids causing a release of enzymes in a dose-dependent manner as observed by Briggs and Briggs [11]. Since high levels of steroids are found in both chronic liver disease [12] and in the second and third trimesters

of pregnancy, elevated levels of these could be a common reason for the increase of lysosomal enzymes in both conditions. A combined effect causing macrophage activation [13] as well as lysosomal membrane labilization is possible.

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Björn Hultberg,

Department of Clinical Chemistry,

University Hospital,

S-221 85 Lund (Sweden)

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Uptake of β -Hexosaminidase by Nonparenchymal Liver Cells and Peritoneal Macrophages

Anders Isaksson^a, Björn Hultberg^a, Roger Sundler^b, Björn Åkesson^a

^aDepartment of Clinical Chemistry, University Hospital, Lund;

^bDepartment of Physiological Chemistry, University of Lund, Sweden

Key Words. Enzyme uptake · β -Hexosaminidase · Macrophages · Nonparenchymal liver cell · Receptor

Abstract. The uptake of β -hexosaminidase (EC 3.2.1.30) in nonparenchymal liver cells (i.e. endothelial and Kupffer's cells) and peritoneal macrophages has been determined by an enzymatic assay. A considerable uptake was noted in nonparenchymal liver cells, whereas no measurable uptake was seen in peritoneal macrophages. The endothelial cells were more active in the uptake of β -hexosaminidase than were the Kupffer's cells. The uptake of β -hexosaminidase by nonparenchymal liver cells showed saturation kinetics and was competitively inhibited by mannan. These findings support the concept that a cell surface receptor on nonparenchymal liver cells mediates uptake of β -hexosaminidase and suggests a difference in the receptor mechanisms on liver and peritoneal macrophages.

Introduction

Infused glycoproteins with terminal mannose (Man) or N-acetylglucosamine (GlcNAc) residues as well as various human and rat lysosomal hydrolases (e.g., β -glucuronidase, β -hexosaminidase, β -galactosidase, α -fucosidase, α -mannosidase) are rapidly cleared from rat circulation [1, 2]. The infused lysosomal hydrolases and glycoproteins are predominantly recovered in the liver. Cell fractionation techniques, autoradiog-

raphy and histochemical studies of rat liver tissue have revealed that the nonparenchymal cells are the principal cells engaged in the recognition of terminal Man or GlcNAc residues [2–4]. The presence of cell membrane receptors for these exposed residues have been postulated to explain this specific and rapid clearance. Protein complexes which appear to be receptors mediating clearance have been isolated from rabbit [5] and rat [6] liver membranes. Rat alveolar, peritoneal and bone marrow derived macrophages in addi-



tion to nonparenchymal liver cells specifically bind glycoproteins with terminal Man or GlcNAc in vitro [7-9]. Competition experiments have shown that lysosomal hydrolases and such glycoproteins compete for binding and uptake [7, 10, 11]. Thus, mononuclear phagocytes have been proposed to be an important component of the in vivo clearance pathway for these glycoproteins. Studies on eviscerated rats after hepatectomy have shown that glycoproteins with terminal Man or GlcNAc and lysosomal hydrolases are cleared from circulation, although considerably slower than in the intact rat, indicating the presence of some clearance outside the liver [1, 12]. Uncertainties remain, however, regarding the capacity of different cells to bind and internalize Man or GlcNAc terminated glycoproteins and concerning clearance mechanisms in vivo. Because of these uncertainties we have used an enzymatic assay to study the uptake of the lysosomal hydrolase, β -hexosaminidase, in cultured nonparenchymal liver cells and peritoneal macrophages.

Material and Methods

Material

Tissue culture media were from Flow Laboratories (Irvine, Scotland, UK). Collagenase type I, mannan and diaminobenzidine were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Metrizamide was from Nyegaard and Co. (Oslo, Norway). Con A-Sepharose and Sephadex G-150 was from Pharmacia (Uppsala, Sweden). DEAE cellulose (Whatman D-52) was obtained from W. and R. Balston (Maidstone, Kent, UK). *p*-Nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside and the 4-methyl-umbelliferyl substrates for enzyme assays were from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., UK). Thioglycolate medium was from Difco Laboratories (Detroit, Mich., USA). Male Sprague-Dawley rats and female NMRI mice were provided by Anticimex (Stockholm, Sweden).

Enzyme Assay

Enzyme assay of β -hexosaminidase (*p*-nitrophenyl substrate) and α -mannosidase (4-methylumbelliferyl substrate) were performed as previously described [13]. Determination of acid phosphatase activity was performed by incubation of 50 μ l 1 mmol/l 4-methylumbelliferyl phosphate, 25 μ l 1 mol/l sodium citrate buffer, pH 5.0, and 25 μ l enzyme sample (culture medium or cell homogenate) for 0, 30 or 60 min at 37 °C. The reaction was stopped by the addition of 3 ml of 0.2 mol/l glycine buffer, pH 10.7, and fluorescence was measured in an Aminco-Bowman spectrofluorimeter with an excitation wavelength of 365 nm and measurement of emission at 448 nm. 4-Methylumbelliferone in glycine buffer was used as standard. β -Glucuronidase was assayed with 100 μ l 1 mmol/l 4-methylumbelliferyl glucuronide in 0.1 mol/l citrate-phosphate buffer, pH 4.5, and 25 μ l enzyme sample. Incubation times were 0, 30 or 60 min at 37 °C with interruption of the reaction as described above. 1 U of enzyme activity represents 1 μ mol substrate hydrolysed per minute. Protein was determined by the method of Lowry et al. [14].

Preparation of β -Hexosaminidase

10-gram samples of human liver obtained at necropsy and stored at -70 °C was homogenized in 40 ml 10 mmol/l sodium phosphate, pH 6.0, and subjected to ultracentrifugation (100,000 g, 30 min). The supernatant was applied to a Con-A-Sepharose column (2.6 $\times$ 8 cm). The column was washed with 70 ml 10 mmol/l sodium phosphate, pH 6.0, eluted with 10 mmol/l sodium phosphate, pH 6.0, containing 350 mmol/l methyl- α -mannopyranoside and 3-ml fractions were collected. The fractions containing β -hexosaminidase activity were pooled, dialyzed against 10 mmol/l sodium phosphate, pH 6.0, and lyophilized. This enzyme preparation was dissolved in the same phosphate buffer and applied to a Sephadex G-150 column (1.5 $\times$ 68 cm) and eluted with 10 mmol/l phosphate buffer. Fractions with β -hexosaminidase activity were pooled and applied to a DEAE cellulose column (0.9 $\times$ 14 cm). After washing with 30 ml of 10 mmol/l sodium phosphate, pH 6.0, elution was performed using a linear gradient mixed from 100 ml of 10 mmol/l sodium phosphate, pH 6.0, and 100 ml of the same buffer containing 0.3 mol/l NaCl. β -Hexosaminidase B was unabsorbed and β -hexosaminidase A was eluted with the gradient. The fractions containing enzyme activity were lyophilized, dissolved in 1 ml

sodium phosphate, pH 6.0, and dialyzed against the same buffer over night. β -Hexosaminidase A and B were both purified to a specific activity of 3,000 U/g protein.

Isolation of Peritoneal Macrophages

Mouse peritoneal macrophages were prepared by a modification of the procedure of Cohn and Benson [15] from outbred female albino mice. Resident peritoneal cells were harvested in 4 ml of Dulbecco's medium containing 1% heat-inactivated fetal calf serum and heparin (20 units/ml) and were plated ($2-8 \times 10^6$ cells/35-mm well) onto plastic 6-well Linbro Tissue culture dishes. Thioglycollate stimulated cells were obtained as above 4 days after intraperitoneal injection of 1 ml of thioglycollate medium. The cells were incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C. Nonadherent cells were removed 2-3 h after plating. More than 90% of the adherent cells were judged as viable macrophages by displaying the following characteristics: (1) They were large, well-spread mononuclear cells which excluded trypan blue and which avidly ingested latex beads (0.81- μ m diameter) and India ink (added at 10 μ l/ml of medium). (2) Attachment to culture dishes was trypsin resistant (0.025% trypsin for 2 h). (3) On exposure to concanavalin A (0.5-20 μ g/ml of medium), numerous cytoplasmic vacuoles appeared within 30 min. (4) Dividing cells were not observed during culture for up to 7 days plating at low cell density (approximately 10⁵ cells/35-mm dish).

Rat peritoneal macrophages were obtained as described above, except that peritoneal cells were harvested in 15 ml of medium.

Isolation of Nonparenchymal Liver Cells

Method described by Seglen [16] and Steer et al. [10] were used with minor modifications for isolation of nonparenchymal liver cells from male Sprague-Dawley rats fed a balanced diet ad libitum. A cell suspension was obtained by perfusion of livers with Hanks' buffer containing 40 mol/l Hepes (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) and 0.05% collagenase. Most of the hepatocytes in the cell suspension were removed by low speed centrifugation (40 g, 1 min) at room temperature. The supernatant was layered over a solution of metrizamide with a density of 1.089 g/cm³ and centrifuged (3,000 g, 45 min) at 4 °C. Purified nonparenchymal liver cells con-

sisting of Kupffer's and endothelial cells were recovered by pipette from the metrizamide supernatant interface. The cells were washed twice with Hanks' buffer and thereafter suspended in Dulbecco's medium containing 10% (v/v) heat-inactivated fetal calf serum. Before plating onto plastic 6-well (35 mm) Linbro Tissue Culture dishes, the cells were counted by a hemocytometer. The number of cells seeded per well varied between 5 and 15×10^6 . After incubation for 2-3 h in a humidified atmosphere of 5% CO₂ in air at 37 °C, nonadhering cells were aspirated. The adherent cells consisted of approximately equal numbers of Kupffer's and endothelial cells as judged by peroxidase activity [17]. Contamination by hepatocytes was less than 2%. Purified Kupffer's and endothelial cells could be selectively enriched by extensive washing of the nonparenchymal cell cultures. Of the adherent cells more than 90% showed peroxidase activity and were thus designated Kupffer's cells. The cells removed by the washing procedures were seeded on new plates and consisted of approximately 80% endothelial cells and 20% Kupffer's cells according to the above criterion.

Cell Culture and Uptake Studies

In the uptake studies nonparenchymal cells and macrophages were cultured in serum-free Dulbecco's culture medium with added penicillin and gentamicin. β -Hexosaminidase was added to final concentrations of 5-700 mU/1.3 ml medium and 100- μ l aliquots of medium were drawn after 10, 60 and 180 min. After 360 min, the remaining medium was aspirated. The cells were scraped from the dishes, harvested in 1 ml of distilled water and stored at -70 °C until analysis (not more than 1 week). No loss of enzyme activity was seen during this period. The cells were thawed and homogenized before determination of enzyme activities and protein content.

Uptake of β -hexosaminidase was calculated from the difference in enzyme activities in cells that had been incubated in the presence and absence of enzyme as well as from differences in enzyme activities in media at time 0 and 360 min. In the uptake studies β -hexosaminidase (A and B), purified as described above with and without the Con A purification step were used. Uptake of β -hexosaminidase B (3,000 U/g protein) was subjected to quantitative study. Inhibition of the uptake of this enzyme preparation was also studied after simultaneous addition of the enzyme and inhibitor (mannan) to the cell medium.

Results

Acid Hydrolases in Nonparenchymal Liver Cells and Peritoneal Macrophages

Among the acid hydrolases assayed in cell homogenates, β -hexosaminidase, β -glucuronidase and acid phosphatase were found in significantly higher concentrations in thioglycollate stimulated macrophages than in unstimulated macrophages or nonparenchymal liver cells (table I). No changes in cellular enzyme activities were seen when the cell density (0.1–1 mg protein/cell culture) or the time of harvest after plating (2–8 h for nonparenchymal liver cells and 2–24 h for macrophages) was varied. The cellular concentration of no other enzyme than β -hexosaminidase increased after culture in medium containing exogenous β -hexosaminidase. The addition of mannan or heat-inactivated (60 °C, 30 min) enzyme to culture medium did not influence the cellular enzyme activities.

Secretion of β -Hexosaminidase

Cells in culture normally release lysosomal hydrolases to culture medium. Therefore, to determine uptake of β -hexosaminidase from medium the spontaneous secretion of this enzyme by the cells in culture was first studied. Nonparenchymal cells and unstimulated macrophages released negligible amounts of the enzyme, whereas thioglycollate stimulated macrophages showed a considerable secretion of enzyme (corresponding to 50–80% of the cellular content) under the duration of the experiment (fig. 1). The addition of mannan or heat-inactivated enzyme (60 °C, 30 min) had no influence on the secretion.

Uptake of β -Hexosaminidase

After addition of β -hexosaminidase B to cultures of nonparenchymal liver cells a continuous decrease of enzyme activity in the culture medium was seen (fig. 2). This decrease was more pronounced as cell density

Table I. Acid hydrolases in macrophages and nonparenchymal liver cells

	β -Hexosaminidase EC 3.2.1.30	α -Mannosidase pH = 4.5 EC 3.2.1.24	α -Mannosidase pH = 5.5 EC 3.2.1.24	β -Glucuronidase EC 3.2.1.31	Acid phosphatase EC 3.1.3.2
Nonparenchymal liver cells, n = 12	34 (25–42)	2.1 (1.4–2.7)	2.7 (2.2–3.7)	12 (9–15)	19 (14–26)
Rat macrophages n = 7	28 (21–36)	6.6 (4.3–7.8)	5.9 (4.2–7.4)	8.3 (6.1–9.7)	3.9 (3.5–5.0)
Mouse macrophages n = 12	44 (30–72)	4.3 (2.8–5.2)	3.1 (2.4–4.7)	5.8 (4.0–7.1)	2.8 (1.2–3.9)
Thioglycollate stimulated mouse macrophages n = 11	190 (120–330)	3.9 (2.1–6.0)	3.1 (2.1–5.3)	23 (11–30)	14 (5–28)

Enzyme activity (mean and range) is expressed as mU/mg of cell protein.

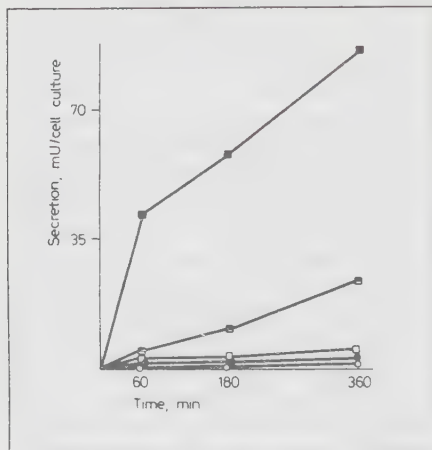


Fig. 1. Secretion of β -hexosaminidase. β -Hexosaminidase released into the medium was determined at different times of incubation and represent milliunits released per cell culture. Cultures of thioglycolate-stimulated macrophages contained 0.2 (□), 0.6 (■) and 1.0 (●) mg protein per dish. Cultures of nonparenchymal liver cells or unstimulated peritoneal macrophages contained 0.2 (○) and 1.0 (●) mg protein per dish.

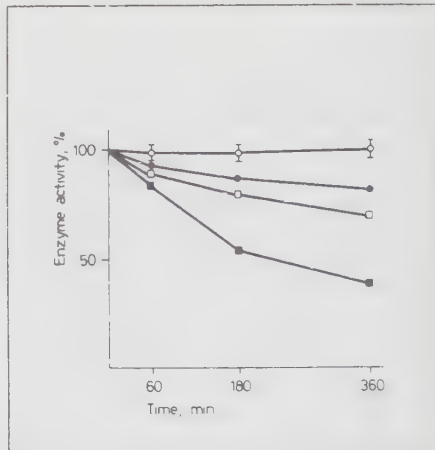


Fig. 2. Uptake of β -hexosaminidase. Nonparenchymal liver cells and peritoneal macrophages were incubated for 360 min with 150 mU of β -hexosaminidase added to culture medium. Enzyme activity remaining in the medium is expressed as a percentage of added activity. Data for nonparenchymal liver cells represent three different cell densities i.e. 0.25 (○), 0.4 (□) and 1.0 (■) mg protein per dish whereas the results for macrophages represent the mean from 10 separate cultures containing 0.2–1.0 mg protein per dish (○).

was increased (fig. 2). The cellular content of β -hexosaminidase increased linearly with time (fig. 3) and the increase in cells appeared to approach saturation with increasing amounts of enzyme added (fig. 4). In the experiments where a large amount (more than 300 mU) of enzyme was added, there was an approximately tenfold increase of cellular enzyme activity during the 6 h of incubation. Uptake measured as decrease of medium enzyme activity agreed well with the increase of cellular enzyme activity.

Peritoneal macrophages from mouse and rat in contrast to nonparenchymal cells showed no detectable uptake of β -hexos-

aminidase (fig. 2). Some minor differences in treatment of the two cell types occurred during isolation (i.e. exposure of peritoneal macrophages to heparin and nonparenchymal liver cells to collagenase). Converse treatment of nonparenchymal cells and macrophages with heparin and collagenase, respectively, prior to studies of enzyme uptake, however, demonstrated no differences in their primary uptake characteristics.

Purified Kupffer's cells and approximately 80% pure endothelial cells were tested separately for uptake of β -hexosaminidase. Both cell types showed a considerable increase of cellular enzyme activity after 6 h of

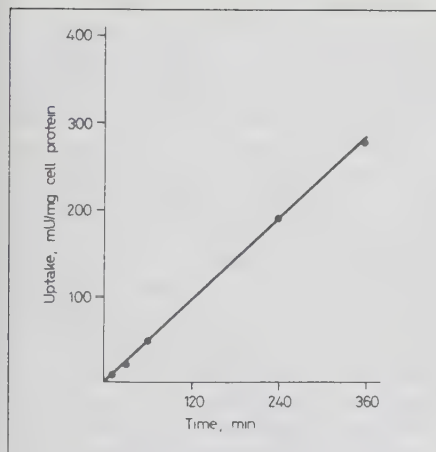


Fig. 3. Uptake of β -hexosaminidase as a function of time. Nonparenchymal cells were incubated with 350 mU of β -hexosaminidase added to culture medium. At the appropriate times the cultures were harvested and β -hexosaminidase activity was determined in the cells. The uptake of β -hexosaminidase expressed as milliunits per milligram cell protein was estimated by comparing each culture with added β -hexosaminidase to a matched control without added β -hexosaminidase.

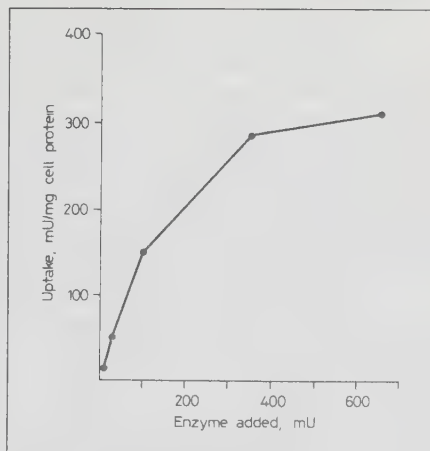


Fig. 4. Uptake of β -hexosaminidase as a function of enzyme concentration. Nonparenchymal cells were incubated for 360 min with the addition of various amounts (5–700 mU) of β -hexosaminidase. Uptake was calculated from the difference in cellular enzyme activity between cells incubated in the presence and absence of enzyme and is expressed as milliunits per milligram cell protein.

incubation in the presence of exogenous β -hexosaminidase. The endothelial cells, however, showed a 3- to 4-fold greater increase (per milligram of cell protein) of cellular enzyme activity than did the Kupffer's cells.

Uptake of the other enzymes studied (β -hexosaminidase A and B) showed qualitatively similar results.

Inhibition of Enzyme Uptake

Mannan is a potent inhibitor of cellular binding and of the clearance of glycoproteins with terminal Man and GlcNAc residues *in vivo*. At all concentrations of β -hexosaminidase tested, the uptake of enzyme by nonpa-

renchymal cells was markedly inhibited by the addition of mannan. The addition of mannan at 5 mg/ml inhibited by 98% the cellular uptake of β -hexosaminidase (fig. 5).

Discussion

In the present study there was a considerable uptake of β -hexosaminidase by the nonparenchymal cells. The results support the concept that nonparenchymal cells internalize β -hexosaminidase by a receptor-mediated endocytosis. This conclusion is based on the following observations: (1) The cellular in-

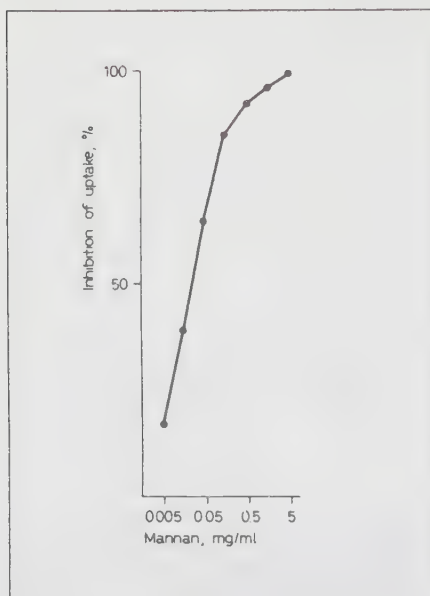


Fig. 5. Inhibition of uptake. Nonparenchymal cells were incubated with 350 mU of β -hexosaminidase and various amounts of mannan. The percentage inhibition of uptake by mannan was estimated by comparing each culture containing mannan to a matched control without mannan.

crease of β -hexosaminidase was linear with time. (2) The increase of enzyme content in cells corresponded to the decrease found in culture medium. (3) None of the other acid hydrolases measured showed any cellular increase. Thus, the increase of cellular β -hexosaminidase cannot simply be explained as a nonspecific stimulus of the lysosomal apparatus. (4) The cellular increase showed saturation kinetics. (5) The increase was competitively inhibited by mannan.

Peritoneal macrophages in contrast to nonparenchymal cells did not show any de-

tectable uptake of β -hexosaminidase. Variations in the method of enzyme preparation (β -hexosaminidase A and B) or treatment of the cells with heparin, collagenase or thioglycollate prior to uptake studies did not alter the results.

Several studies have been published during recent years on the uptake of lysosomal hydrolases. *Stahl* et al. [7] have demonstrated binding of β -glucuronidase to rat alveolar macrophages. Their study was performed as a short time assay (30–90 min) with cells in suspension and radioiodinated enzyme as ligand. Binding of β -hexosaminidase A to nonparenchymal rat liver cells was found by *Steer* et al. [10] using similar techniques to those of *Stahl*. *Kusiak* et al. [8] reported binding of radiolabelled β -hexosaminidase A to rat peritoneal macrophages in culture. In a study by *Ulrich* et al. [11] cultured nonparenchymal rat liver cells were tested for uptake of various lysosomal hydrolases. Significant uptake was noted for several of the hydrolases studied, including β -hexosaminidase. In their study, uptake was measured by specific enzyme assays. The results of these studies are difficult to compare as each group has studied only one cell type and experimental methods, in particular preparation and characteristics of enzymes used have varied. Nonetheless, results suggest similarities in nonparenchymal liver cells and peritoneal macrophages with respect to their uptake of lysosomal hydrolases and possibly other glycoproteins. In the present study using both cell types, uptake of β -hexosaminidase was studied under identical experimental conditions. Several explanations for the discrepancy between the present and earlier studies on the uptake by macrophages are possible. Although our method uses a specific enzyme assay it also requires correction for endoge-

nous activity, which may decrease sensitivity. A slight uptake of the enzyme by peritoneal macrophages may not have been detected by our methods. Another explanation may be that earlier uptake studies on macrophages have been performed exclusively using highly purified radioactively labelled hydrolases as ligands. Slight alterations in the characteristics of the ligands secondary to purification and/or radiolabelling procedures may occur and vary with methods used. Such alterations may affect uptake [18]. The difference in uptake in nonparenchymal liver cells and peritoneal macrophages as well as the observation that antibodies raised against the receptor purified from rat liver did not inhibit the binding of mannose-albumin to rat alveolar macrophages, although they inhibited the binding to the receptor itself [6], suggest that receptors for exposed mannosyl and GlcNAc residues on the two cell types are not identical.

Clearance of lysosomal hydrolases in the intact rat occurs mainly in the liver. Aside from the considerable perfusion and large liver mass, this may be explained by the presence of certain cell types with specific receptors in this organ. In the eviscerated rat the infused hydrolases or glycoproteins with terminal Man or GlcNAc residues show a slow plasma clearance and a diffuse distribution with some enrichment in bone and spleen [1, 12]. These *in vivo* results support our findings of a difference in uptake between macrophages and nonparenchymal liver cells. Furthermore, we have found that the endothelial cells isolated from rat liver tissue were the most efficient cell type studied for the uptake of β -hexosaminidase. Electron-microscope autoradiography for study of the carbohydrate recognition systems in rat liver [4] also demonstrated that endothelial cells had the

most efficient uptake of β -glucuronidase and Man or GlcNAc terminal glycoproteins.

Most clearance studies have been performed in the rat. Investigations of enzyme replacement therapy in patients with Tay-Sachs disease have shown that β -hexosaminidase A is removed from circulation with similar kinetics as in the rat [19] and that the infused enzyme is mainly cleared by the liver. This suggests that similar clearance mechanisms operate in rat and man.

Tissue and serum forms of β -hexosaminidase, although they are related, differ in physicochemical properties [20]. *Bearpark and Stirling* [21] have shown that desialylation of serum β -hexosaminidase A is obligate for its rapid removal from rat circulation. The factors (i.e. receptor or organ) involved in the rapid clearance of the desialylated serum form of β -hexosaminidase or the factors regulating the serum levels of the native sialylated serum forms have, however, not yet been further investigated.

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Anders Isaksson,
Department of Clinical Chemistry,
University Hospital,
S-221 85 Lund (Sweden)

METABOLISM OF THE HUMAN SERUM β -HEXOSAMINIDASE ISOENZYMES IN
THE RAT. AN IN VIVO AND IN VITRO STUDY

A Isaksson^a, M Bergenfeldt^b, B Hultberg^{a*}, B Joelsson^b

Departments of ^aClinical Chemistry, and ^bSurgery, University
Hospital, S-221 85 Lund (Sweden)

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* To whom correspondence should be addressed

SUMMARY

Plasma clearance of purified human serum β -hexosaminidase isoenzymes was studied in the rat. The serum β -hexosaminidase isoenzymes (A,B and P) showed a slow clearance in rat circulation compared to their tissue counterparts. After desialylation the clearance rate of all serum isoenzymes increased markedly.

In vitro the uptake of native as well as desialylated β -hexosaminidase isoenzymes was studied in cultured hepatocytes and non-parenchymal liver cells. Desialylated β -hexosaminidase A and B were taken up by the non-parenchymal liver cells, whereas no such uptake was found for β -hexosaminidase P. None of the isoenzymes interacted with the hepatocytes.

INTRODUCTION

Prior in vitro studies have shown that rat alveolar and peritoneal macrophages as well as non-parenchymal cells of rat liver possess a specific, receptor mediated, uptake mechanism for glycoproteins with terminal mannose or N-acetylglucosamine (1,2). Furthermore, infused glycoproteins terminated by mannose or N-acetylglucosamine, including various human and rat lysosomal hydrolases (β -glucuronidase and β -hexosaminidase), are rapidly cleared from plasma by this recognition system (2-4). The clearance in rat is mainly mediated by the liver and confined to the non-parenchymal liver cells (2-4). It has been proposed that the receptor mediated uptake of glycoproteins bearing terminal mannose or N-acetyl-glucosamine is of importance in the regulation of extracellular levels of various glycosylated macromolecules, including lysosomal hydrolases (5).

We have in earlier studies demonstrated an elevation of serum β -hexosaminidase activity in pregnancy, acute ethanol intoxication and in different forms of liver disease (6-9). In all these conditions the serum isoenzyme, β -hexosaminidase P, is the main cause for the increase in total β -hexosaminidase (10). Two hypothesis have been suggested to explain the increased serum β -hexosaminidase activity; either a defective removal from circulation by non-parenchymal liver cells or increased enzyme production by activated macrophages (10).

In an earlier study we found a considerable uptake of human tissue β -hexosaminidase by rat non-parenchymal liver cells but not by rat peritoneal macrophages (11). To improve our understanding of the mechanisms regulating the serum levels of lysosomal enzymes, we have studied the elimination of highly purified and well characterized human serum

β -hexosaminidase isoenzymes after intravenous infusion into rats, as well as the uptake of the enzymes in cultured non-parenchymal liver cells and hepatocytes.

Material

CH-Sepharose 4B, SP-Sephadex C50, Con A-Sepharose were obtained from Pharmacia (Uppsala, Sweden) and DEAE-cellulose (Whatman DE-52) from W. and R. Balston (Maidstone, Kent, UK). Ampholine pH-range 4-6 and 6-8 was purchased from LKB (Stockholm, Sweden). p-Aminobenzyl-1-thio-2-acetamido-2-deoxy- β -D-glucopyranoside, collagenase type I and IV as well as insoluble neuraminidase type VI were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Metrizamide was purchased from Nyegaard and Co. (Oslo, Norway) and p-Nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside from Koch-Light Laboratories Ltd (Colnbrook, Bucks., UK). Tissue culture media were from Flow Laboratories (Irvine, Scotland). Male Wistar rats were provided by Anticimex (Stockholm, Sweden).

Analytical techniques

Assay of β -hexosaminidase (EC 3.2.1.30) activity was performed as described previously (12) with p-nitro-phenyl-2-acetamido-2-deoxy- β -D-glucopyranoside as substrate. One unit (U) of enzyme activity represents one μ mol substrate split per minute. Protein was determined by the Lowry method (13).

Isoelectric focusing was performed in a 110 ml column, using ampholine with a pH-range of 4-6 or 6-8. The time for electrofocusing was 42h. At the end of the experiment the voltage and current were 550V and 0,8 mA, respectively.

Purification of serum β -hexosaminidase (Table 1)

An affinity column for β -hexosaminidase was prepared by coupling p-aminobenzyl-1-thio-2-acetamido-2 deoxy- β -D-glucopyranoside to CH-Sepharose 4B according to the manufacturer's instructions.

First and second trimester pregnancy serum was dialysed against 10mM sodium phosphate pH 6,0 overnight. Precipitated material was removed by centrifugation. One hundred ml of dialyzed serum were applied to the affinity column (2,9x7,5 cm) on each run. The column was washed with 500 ml 10 mM sodium phosphate pH 6,0. β -Hexosaminidase B and P were eluted with 0,2 M NaCl, whereas β -hexosaminidase A was eluted with 0,5 M NaCl in the phosphate buffer.

The fraction containing β -hexosaminidase B and P was concentrated on a PM-10 membrane (Amicon Corporation), dialyzed against 10 mM sodium phosphate pH 6,0 overnight and applied to a DE-52 column (1,9x18 cm). β -Hexosaminidase B was unabsorbed, whereas β -hexosaminidase P was eluted in a salt gradient mixed from 200 ml phosphate buffer and 200 ml 0.3 M NaCl in the same buffer.

β -Hexosaminidase B from 2 runs was directly applied to a SP-Sephadex C-50 column (0,9x10 cm) equilibrated in 10 mM sodium phosphate pH6,0 to which it was absorbed. The column was washed with 40 ml 10 mM phosphate buffer, whereafter elution was performed with a 200 ml salt gradient (0-0,3 M NaCl).

β -Hexosaminidase P from 2 runs was titrated to pH 5,7 with 20 mM citric acid and put on a SP-Sephadex C-50 column (0,9x10 cm) equilibrated in 10 mM citrate-phosphate buffer pH 5,7. The column was washed with 40 ml of buffer, whereafter the bound enzyme was eluted with a 200 ml salt gradient (0-0,5 M NaCl).

β -Hexosaminidase A from the affinity column was concentrated on a PM-10 membrane, dialyzed against 10 mM sodium phosphate pH 6,0 and applied to a DE-52 column (0,9x20 cm) equilibrated in the phosphate buffer. The column was washed with 60 ml of buffer whereas elution was achieved with a salt gradient (0-0,3 M NaCl). Fractions containing β -hexosaminidase activity were pooled and dialyzed against 10 mM sodium phosphate pH 6,0 overnight. After dialysis the enzyme solution was titrated to

pH 4,8 with 20 mM citric acid and applied to a SP-Sephadex column (0,9x20 cm) equilibrated in 20 mM sodium citrate pH 4,8. The enzyme was bound to the column. The column was washed with 60 ml citrate buffer. The enzyme was eluted in a 200 ml salt gradient (0-0,5 M NaCl) in the citrate buffer.

Purification of tissue β -hexosaminidase

Tissue β -hexosaminidase was purified from human liver as earlier described (11). The specific activity of tissue β -hexosaminidase A and B was about the same as for the serum isoenzymes after the SP-Sephadex step.

Neuraminidase treatment

One ml of enzyme solution (serum β -hexosaminidase A, B and P) was incubated with 0,20 units of insoluble neuraminidase in citrate-phosphate buffer pH 5,0 at room temperature. After 6 h the neuraminidase was removed by centrifugation. The effect of neuraminidase treatment was controlled by isoelectric focusing.

Infusion of β -hexosaminidase

Thirty-six male Wistar rats (180-250g) were used. They were anesthetized with chloralhydrate 5% (0,5 ml/100g body weight) and the anesthesia was stabilised with ether. A cannula was inserted in the femoral vein and used for injection of the enzyme bolus (1 ml; 100-150 mU; peak activity in rat circula-

tion then was at least the double basal level) or 0,9% NaCl (controls) and also for the subsequent blood sampling (500 μ l each time). In experiments using tissue β -hexosaminidase isoenzymes, 400-500 mU were infused. Blood samples were obtained immediately before the enzyme injection and after 2, 10, 30, 60 and 120 min. The cannula was flushed with saline after every sampling to maintain the blood volume of the rat. Heparin plasma was stored at -20°C until analysis.

In 8 rats 2 cannulas were used. One cannula was inserted in the jugular vein and used for injection of the enzyme. Another cannula was inserted in the femoral vein and used for blood sampling. As no difference was found in the results between the two methods the simpler procedure with only one femoral vein cannula was preferred.

Cell isolation

Isolation of hepatocytes and non-parenchymal liver cells was performed as described previously (11).

Cell culture and uptake studies

Cells were cultured in Medium 199 with 5% fetal calf serum containing penicillin and stryptomycin. Non-parenchymal liver cells were plated onto plastic 6-well (35 mm) Linbro Tissue culture dishes. The number of cells seeded per well varied between 5 and 10×10^6 . After incubation for 2-3 h in a humidi-

fied atmosphere of 37°C , nonadhering cells were aspirated and the plates were washed 3 times with phosphate buffered saline. The cells were incubated in serum-free medium containing 20-70 mU of the different serum isoenzyme forms. After 6 h the medium was aspirated. The cells were scraped from the dishes, harvested in 1 ml of distilled water and stored at -20°C until analysis. The cells were thawed and homogenized before determination of enzyme activity and protein content.

Uptake was calculated from the difference in enzyme activity in cells that had been incubated in the presence and absence of enzyme as well as from differences in enzyme activity in media at 0 and 6 h.

Hepatocytes ($1-2 \times 10^6$) were seeded onto collagen coated dishes and were used for study of enzyme uptake the next day. Otherwise, the procedure was the same as for the non-parenchymal cells.

RESULTS

Intravenous infusion of β -hexosaminidase

Whereas tissue β -hexosaminidase A was almost completely cleared from circulation within 10 min, all serum forms exhibited a much slower clearance (Fig 1). Tissue β -hexosaminidase B behaved as tissue form A (not shown). Serum isoenzymes purified only to the DE-52-step (specific activity 100-200 u/g protein)

showed the same disappearance rates as the more highly purified serum isoenzymes (not shown). Following desialylation all serum isoenzymes quickly disappeared from circulation after infusion. The peak value of β -hexosaminidase activity found in plasma samples after infusion was only about 20% of that expected with regard to the amount of enzyme infused (Fig 1).

Uptake of β -hexosaminidase in vitro

Neither in hepatocytes nor in non-parenchymal liver cells there was any detectable uptake of the native serum isoenzymes.

Uptake of desialylated β -hexosaminidase A and B was found in the non-parenchymal liver cells, whereas no such uptake was found for desialylated β -hexosaminidase P. (Table 2). In the hepatocytes no uptake of any of the desialylated serum isoenzyme forms was seen .

DISCUSSION

Our results demonstrate that the native serum forms of β -hexosaminidase are eliminated much slower than the tissue forms. These findings are in accordance with those of Bearpark and Stirling (14) who have shown that purified (the degree of purification is not stated) native human serum forms of β -hexosaminidase exhibit a much slower elimination from rat circulation compared to the tissue forms.

Likewise, Desnick et al (15) have demonstrated the same pattern of elimination of another lysosomal enzyme, α -galactosidase, purified from plasma and spleen, respectively. In fact, plasma α -galactosidase exhibits about the same half-life time (1 - 2 hours) in human as human serum β -hexosaminidase isoenzymes in rats.

The clearance mechanism of most native serum enzymes (e.g. native β -hexosaminidase isoenzymes) is still obscure (16) and no particular recognition system has been found. Desialylation of the serum forms obviously increases the clearance rate markedly, which was also noted by Bearpark and Stirling (14) regarding β -hexosaminidase A.

β -Hexosaminidase P post partum in human (17) shows a half-life of about 10 - 15 hours, which is much longer than our findings in rats. This might, however, depend upon a dwindling production post partum resulting in a falsely long half-life.

Zühlsdorf et al (18) have recently demonstrated that most of β -hexosaminidase present in human serum appears to consist of high molecular weight precursors to the mature lysosomal tissue forms. Probably the serum precursor forms have complex oligo-saccharide chains containing sialic acid (18-20). After removal of the sialic acid, the galactose terminals would be uncovered and theoretically the serum forms of β -hexosaminidase would be eliminated via the galactose-receptors on the hepatocytes (21). However, our in vitro studies show that desialylated serum

β -hexosaminidase A and B are taken up only by the non-parenchymal liver cells, whereas the desialylated P form does not interact with any of the cell types investigated. Actually we expected that the behaviour of the isoenzymes should be the same after removal of sialic acid, since Geiger et al (22) have suggested that β -hexosaminidase P only differs from the B form in their content of sialic acid. Our results might be due to interfering substances which have not been eliminated during the enzyme purification or that Hex P is cleared via another cell type.

The mechanism by which serum β -hexosaminidase A and B are taken up by the non-parenchymal liver cells is not obvious but several workers have found that asialoglycoproteins can interact with galactose-receptors found on the non-parenchymal liver cells (23). It might also be that removal of sialic acid makes it possible for the two isoenzymes to be recognized via high mannose oligosaccharides that might co-exist in the enzyme molecule together with the complex oligosaccharides in analogy with glucocerebrosidase and mannosidase (24,25). Another possibility might be uptake via an as yet uncharacterized receptor.

In conclusion native serum forms of β -hexosaminidase are cleared from the circulation (by an unknown mechanism) more slowly than tissue forms of β -hexosaminidase. Desialylated serum enzymes are eliminated much faster than the native serum forms and in vitro experiments makes it likely that at least

β -hexosaminidase A and B are taken up by non-parenchymal liver cells. The fate of β -hexosaminidase P is still unknown.

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TABLE 1 Enzyme purification

	Enzyme	Total activity Units	Total protein g	Spec activity Units/g	Purification -fold	Yield %
Serum		10,2	15,9	0,64	-	-
Affinity	A	2,8	$7,6 \times 10^{-2}$	36,5	152	72
DE-52	A	2,1	$1,9 \times 10^{-2}$	110	458	54
SP-	A	0,79	$0,6 \times 10^{-3}$	1217	5070	20
Affinity	B+P	4,6	$3,4 \times 10^{-1}$	13,5	35	73
DE-52	B	1,2	$5,9 \times 10^{-3}$	202	776	27
SP	B	0,60	$0,7 \times 10^{-3}$	848	3262	14
DE-52	P	0,50	$3,2 \times 10^{-3}$	152	1169	23
SP	P	0,16	$0,1 \times 10^{-3}$	1565	12038	7

Characterization of the isoenzymes into A, B and P is based on their different behaviour when chromatographed on DE-52 at pH 6,0. The purification factors are calculated from a DE-52 chromatography of an aliquot of dialyzed serum sample, where isoenzyme A, B and P constituted 38, 41 and 21 % of total activity.

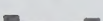
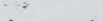
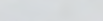
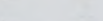
TABLE 2 Uptake of β -hexosaminidase

Enzyme	Uptake in non-parachymal liver cells (mU/mg cell protein)	Uptake in Hepatocytes (mU/mg cell protein)
Native β -hexosaminidase A	ND (n=4)	ND (n=4)
" B	ND (n=4)	ND (n=4)
" P	ND (n=5)	ND (n=6)
Desialylated A	90-110 (n=3)	ND (n=4)
" B	80-110 (n=3)	ND (n=4)
" P	ND (n=4)	ND (n=4)

Uptake of β -hexosaminidase is calculated from the difference in enzyme content (mU/mg cell protein) between cell cultures with and without enzyme added to culture medium. ND = not detected.

LEGENDS

Figure 1. Elimination of β -hexosaminidase from rat circulation. Percent expected activity is equal to percentage of a calculated value based on the amount of enzyme given. For the purposes of these calculations the plasma volume of a 200 g rat was taken to be 7 ml. Median of three to five experiments is presented.

	physiological saline		
	serum β -hexosaminidase A		
	serum	- " -	P
	serum	- " -	B
	tissue	- " -	A
	desialylated serum β -hexosaminidase A		
	desialylated serum	- " -	P
	desialylated serum	- " -	B

Percentage of expected activity

